NEUROBIOLOGY
OF INVERTEBRATES

Symposia Biologica Hungarica

36

NEUROBIOLOGY OF INVERTEBRATES

36

Akadémiai Kiadó, Budapest

NEUROBIOLOGY OF INVERTEBRATES

Edited by

J. SALÁNKI and K. S.-RÓZSA

(Symposia Biologica Hungarica 36)

This volume containing papers of a Satellite Symposium of the 2nd World Congress of Neuroscience covers a wide spectrum of research concerning neurons, brain and behaviour of invertebrate animals. It includes physiology, pharmacology and localization of specific chemical substance, peptidergic mechanisms, problems of modulation, integration and learning, as well as ionic channels and intracellular mechanisms mainly in the brain of molluscs and arthropods. It highlights novel aspects of neurotransmitter research like the interaction of transmitters and modulators, the interrelationship between chemical substances and ionic channels, the significance of intracellular processes during and after transmitter action, and immunocytochemical detection of transmitter localization.

The new results obtained by way of simple model systems add to the basic knowledge applicable in understanding similar processes in the nervous systems of higher animals and man.

The significant interest in these topics is proved also by the fact that representatives of a large number of research laboratories from 15 countries attended the symposium.

AKADÉMIAI KIADÓ
BUDAPEST

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J. SALÁNKI et KATALIN S.-RÓZSA

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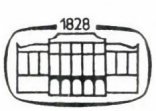
TRANSMITTERS, MODULATORS AND RECEPTORS

Edited by

J. SALÁNKI and KATALIN S.-RÓZSA



AKADÉMIAI KIADÓ, BUDAPEST 1988

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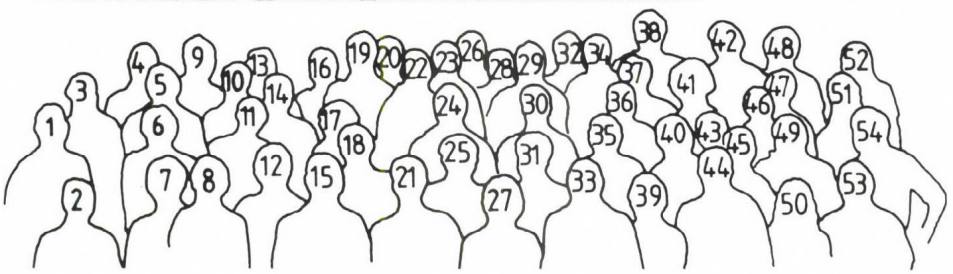

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PREFACE

The first International Symposium on Neurobiology of Invertebrates was organized in Tihany in 1967 followed by five others. The sixth was held in 1987 partly stimulated by the 2nd World Congress of Neuroscience as its Satellite Symposium.

This volume contains all papers of the Symposium dealing with the physiology, pharmacology and localization of low molecular weight neurotransmitters and those of peptides. The questions of modulation, integration and learning, as well as intracellular mechanisms and functioning of ionic channels have also been treated. The great variety of invertebrate species from Ciliata to Arthropoda indicates the comparative aspects of the topics.

Similar to the previous volumes, each paper is followed by discussions including the questions, views and remarks thereby presenting the state-of-the-art of research in this field.

We would like to express our sincere gratitude to members of the Organizing Committee, E. Florey, D.A. Sakharov, G.B. Stefano, L. Tauc and R.J. Walker, for their helpful assistance in organizing and conducting the Symposium. Our thanks are also due to all the participants of the Symposium for their valuable contribution to this volume.

The Editors

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Symposia Biologica Hungarica 36 (1988)

INTRODUCTORY REMARKS

INVERTEBRATES IN NEUROSCIENCE

J. SALÁNKI

Balaton Limnological Research Institute of the
Hungarian Academy of Sciences
Tihany, Hungary

It is a great pleasure and honour to greet the participants of the Symposium, representing invertebrate neurobiologists from 14 countries at the Balaton Limnological Research Institute of the Hungarian Academy of Sciences, in Tihany.

The Symposium was organized as a Satellite of the 2nd World Congress of Neuroscience, taking advantage of the presence of a large number of neurobiologists. We wanted to create a memorable occasion particularly for scientists studying invertebrate nervous systems to gather and report on their latest results and to exchange ideas under these intimate circumstances.

As most of you know, this is not the first conference on invertebrate neurobiology held this Institute called before 1982 Biological Research Institute of the Hungarian Academy of Sciences.

The favourable reaction and the renewed interest of the former participants to these meetings have encouraged us to organize this 6th Invertebrate Neurobiology Symposium in Tihany.

It should be mentioned that this symposium fits into a series of events at the Institute commemorating its foundation in 1927. The present meeting coincides with the sixtieth anniversary, and you are particularly warmly welcomed on this occasion by the directorate, the research staff and the personnel of the Institute.

Over the next few days we plan to talk about results and problems which are in the forefront of our field of research, and to discuss the questions we are currently concerned with. As you know, invertebrate neuroscience studies are specific not

only to these phyla, but in many cases, the results obtained in invertebrates provided a starting point, a key to the understanding of the detailed processes in the nervous system of higher animals including man. It is well known that our knowledge about neurotransmitters regarding both their presence and functioning, has greatly been enriched during the past decade, and this progress is largely based on the results obtained in molluscs, insects, crabs and other lower animals. There are new aspects in neurotransmitter research which were, in many cases, beyond our earlier interest, e.g. the existence of modulators and modulator systems, studies concerned with receptors, the interrelationship between transmitters and ionic channels, the significance of intracellular processes during and after transmitter action, and finally studies concerned with the evolutionary significance of signal systems. The sophistication of intracellular communication is accentuated by the discoveries of similar signal molecules in both the vertebrate and invertebrate nervous systems, such as the opioid family. In addition, the strong protein homologies are noted in the biochemical analysis of the Na-channels of both animal groups.

Certainly the questions raised in the subtitle of our Symposium can be answered today only partially or hypothetically. A more adequate, and well-founded reply will be given only in the future. This is part of the normal scientific process, and this meeting can only be a small step in the sequence of scientific events recording and discussing the latest results.

Those who have carried out or followed ongoing research in the field of neurobiology during the last twenty years may recall a number of examples which prove that the explanation of our scientific observations is not always simple and plausible. In the 60s it was generally accepted that during an action potential the carrier for inward current could only be Na^+. At our first Symposium in Tihany in 1967, two presentations dealt with the fact that in some Helix neurons action potentials are generated in Na-free saline. One of the authors explained this unusual observation by supposing that a Na-layer remains around the neuron even in sodium-free saline, which is responsible for the generation of the action potential; another colleague set

forth the idea that in this case Ca-ions are responsible for the inward current. The programme of the 1975 Symposium, however, offered convincing evidence that Ca-ions also play a role not only in the regulation of membrane permeability, but also as carriers for the inward current along with sodium.

Contradictory explanations were presented at the earlier Tihany Symposia, too, as regards the extra- and/or intracellular regulation of membrane permeability, especially the regulation of bimodal pacemaker activity. Although none of the theories has substiantially been verified, many of the details has been clarified using voltage clamp and pharmacological approaches.

Twenty years ago the transmitter role and the place of intracellular localization of octopamine, histamine and even of serotonin and dopamine were uncertain. This was a question for debate at almost all of our Symposia. Based on electrophysiological, biochemical and morphological investigations, currently, their transmitter function is not questioned. In addition, immunocytochemistry has provided a new and more reliable proof of their cellular localization and identity. Advanced biochemical methodologies have verified the presence of mammalian-like neuropeptides in invertebrates also indicating the presence of invertebrate peptides in vertebrates.

The second messenger role of monoamines is also a recurring question which, it is hoped, will be solved in the future.

Stimulating original experiments were presented here concerning the behaviour-regulating role of identified trigger neurons in the whole animal or in semi-isolated, functioning organ preparates. Based on the visual and electrophysiological identification of neurons, the research on specific interneuronal pathways and neural networks has become possible at the cellular level, the nervous system of invertebrates providing a specific advantage. Although the methods and approaches worked out in molluscs and insects were applied to vertebrates, in the higher animals there are unavoidable difficulties in investigating the same identified units of a circuit from animal to animal.

Our knowledge of 20 years ago concerning neurosecretion has also changed considerably. At that time and even later, there was great controversy on the morphological criteria of identification, while today the investigations aim at the separation, chemical determination, the research on the hormone, mediator or modulator function of peptides first recognized as neurosecretory substances.

The chemical heterogeneity of neurons became generally accepted in the 60s, however, the fact that the same neuron may contain more than one neurotransmitter or modulator substance has only been proved after long detailed experimentation during the recent years, mainly in invertebrates.

Recent research has revealed several well identified nerve cells to be really multifunctional neurons in the brain of molluscs, which are supposed to act as integrative units.

Finally, I should like to mention another issue which has been studied for a long time in invertebrate neurobiology and which was regularly discussed at our symposia. This is the question of learning at the cellular level. Today there is no doubt that invertebrates offer a good model not only for studying learning at well-known neuronal networks of molluscs, but also for investigating the effect of endogenous or exogenous substances influencing this process. This topic is also an important chain linking invertebrate and human neuroscience.

Our functional knowledge of transmitters and endogenous neural mechanisms requires the investigation of processes which are involved in their synthesis, release, decomposition, binding to receptors and their short-term or long-term effect on both pre- and postsynaptic membranes. To answer these question we frequently turn to exogenous chemical substances specifically influencing certain particulate processes of neurotransmission. Some of them, like curare, atropine or TTX are natural products which have toxic effects but which are not encountered in everyday life. At the same time, during the last decades more and more substances have appeared or accumulated in our environment as a result of human activity, which may have an unfavourable effect on living processes. In part, these influences are exerted through the nervous system and

supposedly some of them influence processes associated with transmitter action.

This concept has prompted us to start investigations concerning toxic heavy metals, of which many biological effects are known. Our question was whether they influence the response of the excitable membrane to the known transmitters. It is known that cadmium and cobalt are used for blocking calcium channels. We also have demonstrated the depressing effect of cadmium on the inward and outward currents of Helix neurons. Our present investigation on the neurons of Helix, Lymnaea and Aplysia isolated brains is concerned with the question whether the response to ACh, 5HT and dopamine changes in the presence of Hg and cadmium-ions. Naturally, only neurons were suitable for investigation which reacted to one or more of these transmitter substances.

The transmitters were applied to the surface of the nerve cell body in short pulses, the toxic heavy metals were added to the whole preparation, while recording the resting and action potentials. Perfusion lasted 20-30 minutes, checking from time to time whether the reactions to the transmitters were the same as in the controls. In one preparation only the effect of mercury or cadmium could be tested, since these metals bind strongly to the membrane and their washing out is only partial even after a long time.

The response to neurotransmitters changed significantly in a number of neurons. The character of change was different, as seen in Figs 1-5.

Figure 1 shows the effect of $CdCl_2$ (10^{-6} mol/l) on the response of the identified RPa2 neuron of Helix pomatia to 5HT. The stimulatory effect evoked by 5HT (A) was reduced 20 min after $CdCl_2$ treatment (D), while the response to ACh remained intact (E).

The same neuron lost its responsiveness to ACh under the effect of $HgCl_2$ (10^{-6} mol/l) (Fig. 2), however, the ability to generate spontaneous action potentials was still present with the same frequency.

In a Lymnaea neuron the response to ACh was elicited only after $CdCl_2$ treatment (Fig. 3) being a stimulatory one.

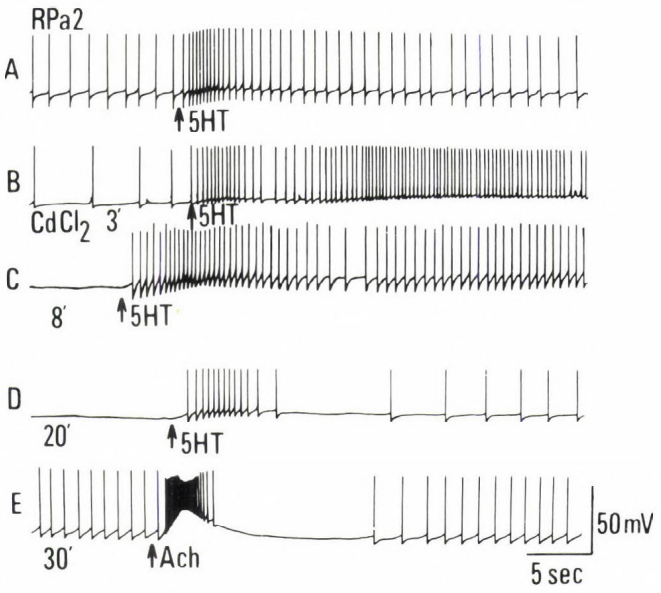

Fig. 1. Modulatory effect of $CdCl_2$ on 5HT response

In another unidentified Lymnaea neuron the inhibitory effect to DA turned to an excitatory one following $CdCl_2$ treatment (Fig. 4).

The variety of effects could be explained by the heavy metal ions probably having a selective effect on the receptors of different neurons or on their ion channels, i.e. on particular processes associated with transmitter action. The following experiment demonstrates this selectivity. The caudodorsal neurons of the pleural ganglia of Aplysia are sensitive to ACh causing hyperpolarization. Inhibition occurs as a result of activation of chloride or potassium channels and there are neurons in which both channels are activated by ACh. At a suitable holding potential, the activation of both channels results in an outward current following ACh application. The Cl current is fast and of short duration, while the potassium current evokes a response which desensitizes slowly.

Figure 5 shows the outward currents in voltage-clamp experiments at -50 mV holding potential. After adding $CdCl_2$, the response changes in such a manner that the Cl component remains

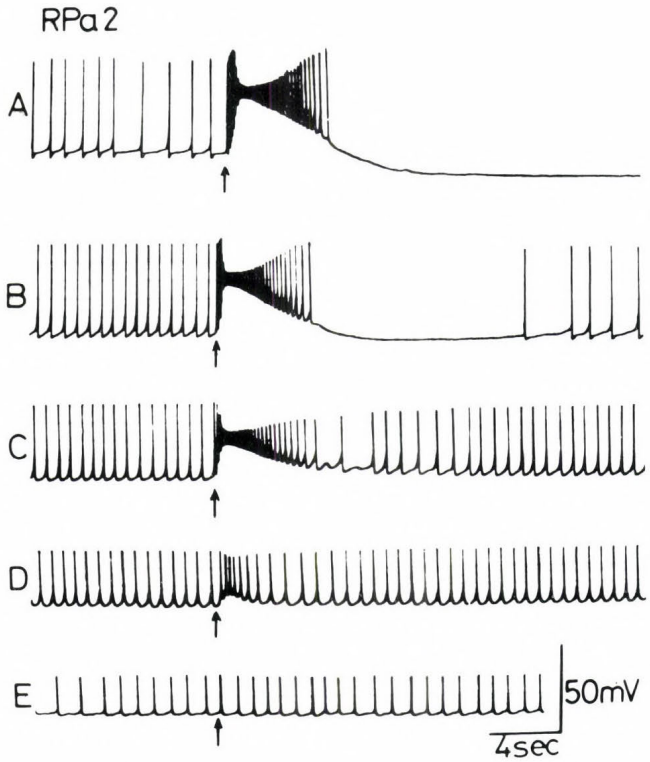

Fig. 2. Modulatory effect of $HgCl_2$ on ACh response

whereas the potassium current is eliminated. The Cl current remains intact, as demonstrated by the fact that it can be reversed at a holding potential greater than -60 mV to an inward current.

In the experiments $CdCl_2$ blocked only the K channels, inhibiting those processes which result in the activation of the K current and it did not influence the activation mechanism of the Cl channel.

In our experiments relatively high amounts, i.e. 10^{-6} mol/l of $CdCl_2$ or $HgCl_2$, were used and they were added directly to the preparations. Similar Cd concentrations are used, however, for the selective blocking of Ca channels. As demonstrated, cadmium is suitable not only for blocking calcium channels. In subsequent studies we should like to clarify whether other heavy metals and toxic substances, which are significant from

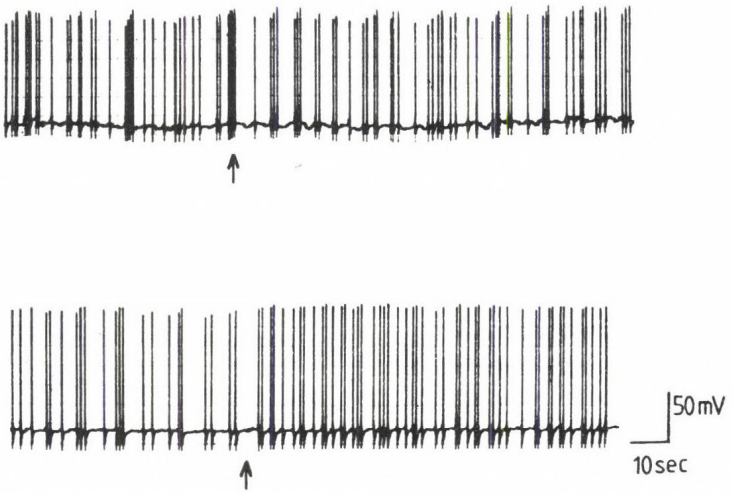

Fig. 3. Sensitization to ACh by $CdCl_2$

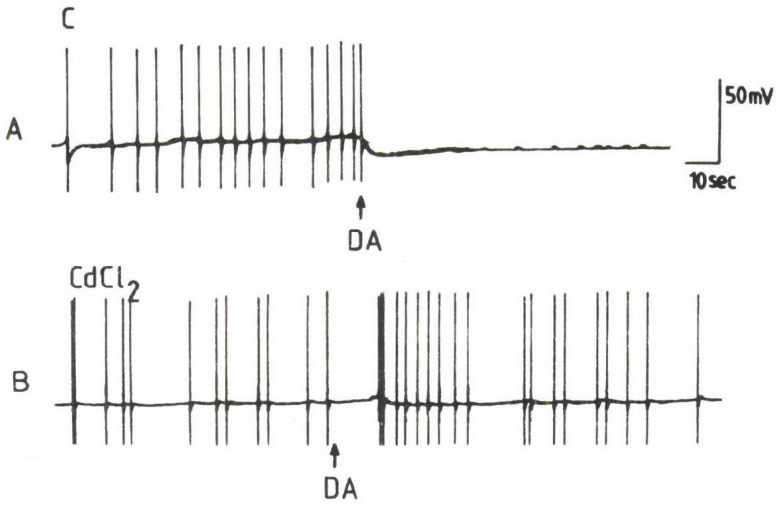

Fig. 4. Reversal of DA effect following $CdCl_2$ treatment

the point of view of environmental pollution, influence the transmitter mechanisms, and if they do, what kind of effect can be ascribed to them? This is done first to obtain information about the effect of environmental pollutants on the mechanisms of neural regulation, which is important from the point of view

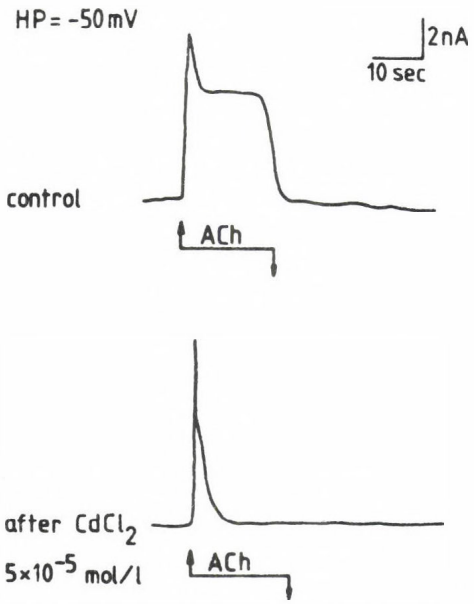

Fig. 5. Effect of $CdCl_2$ on the K current evoked by prolonged application of ACh

of environmental neurobiology and, secondly, because we hope that neurobiology can be enriched with new blockers or modulators suitable for studying specific processes in the action of transmitters/modulators.

I think these two aspects complement each other and are directly linked to the main question of neurobiology, which is always in the focus of our research: the better understanding of neural mechanisms, not only to increase our knowledge on the functioning of the brain, but also to prevent or correct malfunctions, and to utilize its capacity better by improving normal neurological phenomena.

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PHYSIOLOGY, PHARMACOLOGY AND LOCALIZATION

Symposia Biologica Hungarica 36 (1988)

ACETYLCHOLINE RELEASE AT AN IDENTIFIED CENTRAL SYNAPSE OF APLYSIA IS REGULATED BY MUSCARINIC AND NICOTINIC AUTORECEPTORS

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INTRODUCTION

The efficacy of chemical synapses largely depends on the quantity of neurotransmitter released by the presynaptic terminal. The best studied factors regulating are changes in calcium ion influx and intraterminal Ca^{++} ion concentration. Nevertheless, other mechanisms may be involved such as the availability of transmitter within the terminal (Poulain et al. 1986). Activation of presynaptic receptors is another possible physiological way that transmitter release may be modulated. The existence of presynaptic receptors has been most thoroughly described in vertebrates (for review see Chesselet 1984). Concerning cholinergic structures, presynaptic muscarinic acetylcholine autoreceptors have been implicated in a negative feedback loop governing acetylcholine (ACh) release. Their activation by agonists, such as oxotremorine or carbachol, reduces the amount of ACh released, whereas their blockade by atropine induces the opposite effect. Less information is available concerning presynaptic nicotinic acetylcholine autoreceptors (Rowell and Winkler 1984; Briggs and Cooper 1982; Bowman et al. 1984; Wessler et al. 1987). They are thought to be involved in positive feedback control of ACh release as antagonists such as tubocurarine or hexamethonium decrease ACh release.

Only a few studies have suggested the presence of autoreceptors in invertebrates (Woodson et al. 1975; Baux et al. 1986). Taking advantage of identified cholinergic neuro-neuronal synapses in the buccal ganglion of Aplysia californica we have demonstrated the coexistence, on the same terminal, of both muscarinic-like and nicotinic-like autoreceptors (Baux et al. 1987; Baux and Tauc 1987; Poulain et al. 1987).

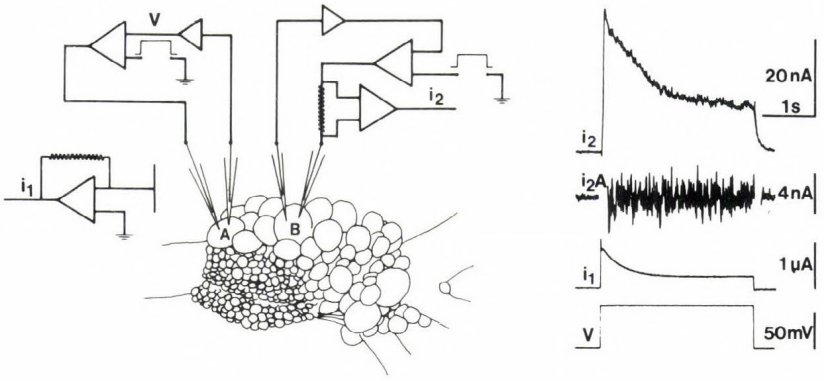


Figure 1: Schematic drawing of the recording circuit (on the left) and the corresponding recordings (on the right).

One of the two presynaptic neurones (A) and one of the postsynaptic neurones (B) were simultaneously voltage clamped at -50 and -80 mV, respectively. A virtual ground gives the sum, i_1, of both pre- and postsynaptic currents. i_2 is the postsynaptic current. The presynaptic current is then given by subtracting i_2 from i_1 (i_2 is negligible compared to i_1). Recordings (right panel): The long duration postsynaptic curent (i_2) was obtained with sustained (3 s) depolarizations (V) of the presynaptic neurone. For the analysis of the synaptic noise, i_2 was recorded at high gain as an a.c. trace (i_2A). The size of the miniature postsynaptic current (MPSC) is given by the relation: $2.E^2/I_m$ where E^2 is the variance of the noise calculated from i_2A, and I_m the mean value of i_2.

METHODS

The isolated and desheathed ganglions were bathed with artificial sea water (ASW) of composition (mM): NaCl, 460; KCl, 10; $CaCl_2$, 11; $MgCl_2$, 25; $MgSO_4$, 28; Tris HCl buffer, 10: pH 7.8. Drugs were used in ASW solutions and applied for at least 20 min before recording.

Quantal ACh release was induced by a 3-second depolarization of the voltage-clamped presynaptic neurone in the presence of tetrodotoxin (10^{-4}M)

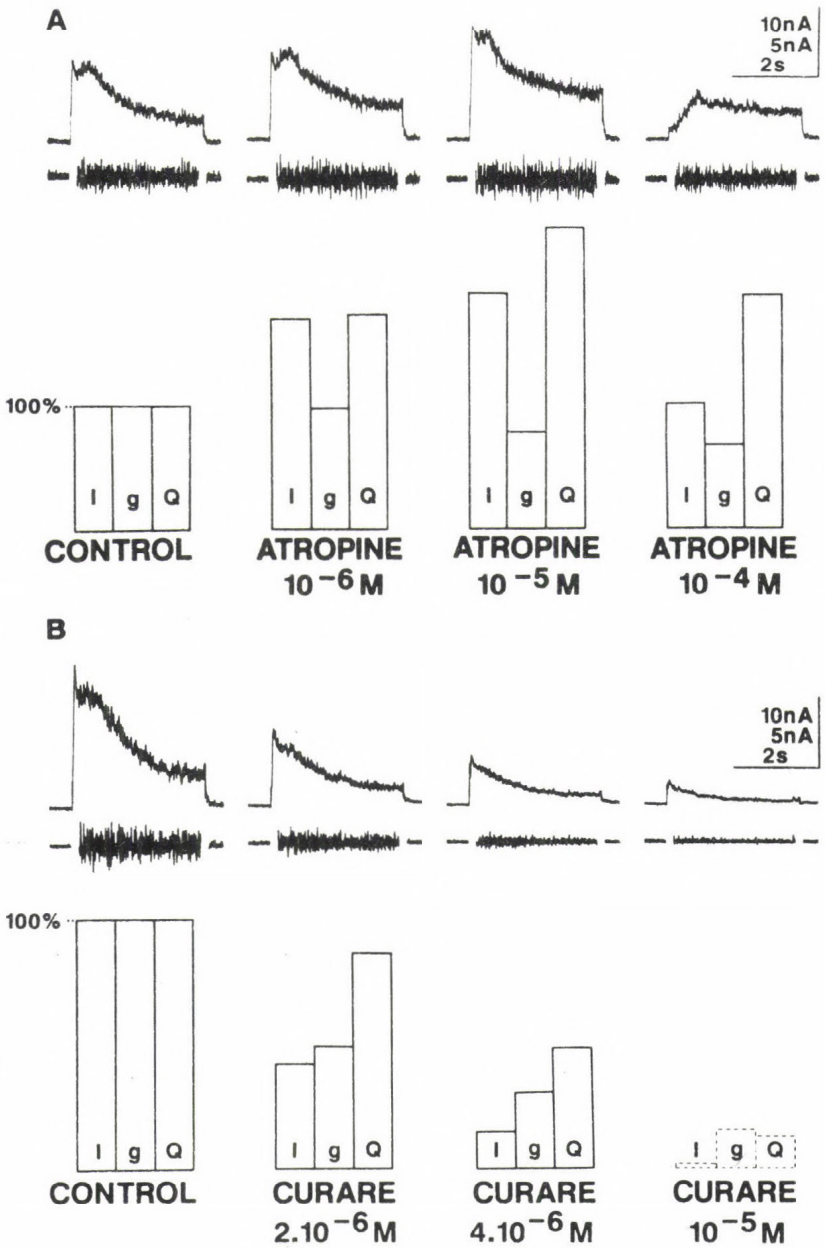
(Simonneau et al. 1980). The long-duration induced postsynaptic current response recorded in the voltage-clamped (-80 mV) postsynaptic neurone displayed synaptic noise at its peak (Fig. 1). Statistical analysis of these responses (Simonneau et al. 1980) allowed calculation of the amplitude and the decay time of miniature postsynaptic currents (MPSCs) that compose the postsynaptic response and correspond to the release of one quantum of ACh. ACh release was estimated by calculating the quantal content of the postsynaptic response (number of quanta released per presynaptic impulse). This was achieved by dividing the quantity of current of a long-duration response by that of the MPSC (Baux et al. 1986, Poulain et al. 1987).

RESULTS

Cholinergic antagonists suggest the presence of presynaptic ACh receptors.

If muscarinic autoreceptors involved in negative feedback control of ACh release exist at the presynaptic terminal, their blockade by an antagonist would lead to an increase in the number of quanta released per stimulus. Accordingly, we have shown (Baux and Tauc 1987; Baux et al. 1987) that atropine, at concentrations between 10^{-6} and 10^{-5}M, increases the size of the postsynaptic response (Fig. 2A). At the same time, the amplitude of MPSCs was decreased (due to weak antagonism by atropine of postsynaptic ACh receptors; Tauc and Gerschenfeld 1962), leading to the conclusion that the number of quanta released was even more enhanced than the response amplitude indicated.

The existence of nicotinic autoreceptors was investigated using antagonists such as tubocurarine (curare) or hexamethonium. Applied at concentrations less than 10^{-5}M in order to prevent total blockade of postsynaptic ACh receptors, curare decreased the postsynaptic response (Fig. 2B). This response was depressed more than was the calculated MPSC amplitude, indicating that, in addition to its classical postynaptic effect, curare has a presynaptic action whereby it decreases the number of ACh quanta released per stimulus. Similarly, application of hexamethonium (a compound that has no effect on postsynaptic H-type ACh receptors coupled to chloride channels) induced a decrease in evoked quantal ACh release. Thus, we have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proposed the existence of nicotinic-like autoreceptors involved in positive feedback control of ACh release (Baux and Tauc 1987; Baux et al. 1987; Poulain et al. 1987).

Activation of muscarinic-like and nicotinic-like ACh presynaptic receptors

Since the above results had suggested the participation of presynaptic muscarinic receptors in negative feedback control of ACh release, compounds generally considered to be muscarinic agonists such as carbachol or pilocarpine could be expected to provoke a decrease in ACh release. In the range of concentrations used, carbachol and pilocarpine had no postsynaptic effect. However, bath applied carbachol or pilocarpine (10^{-8}M to 10^{-4}M) induced a decrease in postsynaptic responses due to a reduction in the number of quanta released (Fig. 3). Moreover, this decrease in evoked ACh release was prevented by atropine but not by curare (Fig. 4) or hexamethonium. The actions of carbachol and pilocarpine can thus be attributed to the activation of presynaptic muscarinic-like ACh receptor.

←

Figure 2: *Effects of atropine and curare on ACh release at an inhibitory cholinergic synapse in the buccal ganglion of Aplysia.*

The recordings (upper trace) show the postsynaptic current responses induced by presynaptic depolarizations to +20 mV during 3 seconds. In A and B, the postsynaptic neurone was voltage-clamped at -80 mV. The a.c. recordings (lower trace) show, at high gain, the fluctuations at the peak of the responses. Histograms represent, with respect to control (100%), the evolution of the mean amplitude of the postsynaptic current response (I), the MPSC (g) and the number of quanta released for the postsynaptic response **(Q)** *after bath application of atropine (A) or curare (B). All postsynaptic currents are calculated as apparent conductances. The quantal content was increased by atropine and depressed by curare. With curare (10^{-5}M) the postsynaptic response was decreased to the extent that estimates of quantal parameters were subject to some error (dashed lines). In A: 100% represent 16930 quanta for* **Q** *, 2.7 nS for g and 167 nS for I. In B: 100% represent 19875 quanta for* **Q** *, 2 nS for g and 159 nS for I (from Baux et al. 1987).*

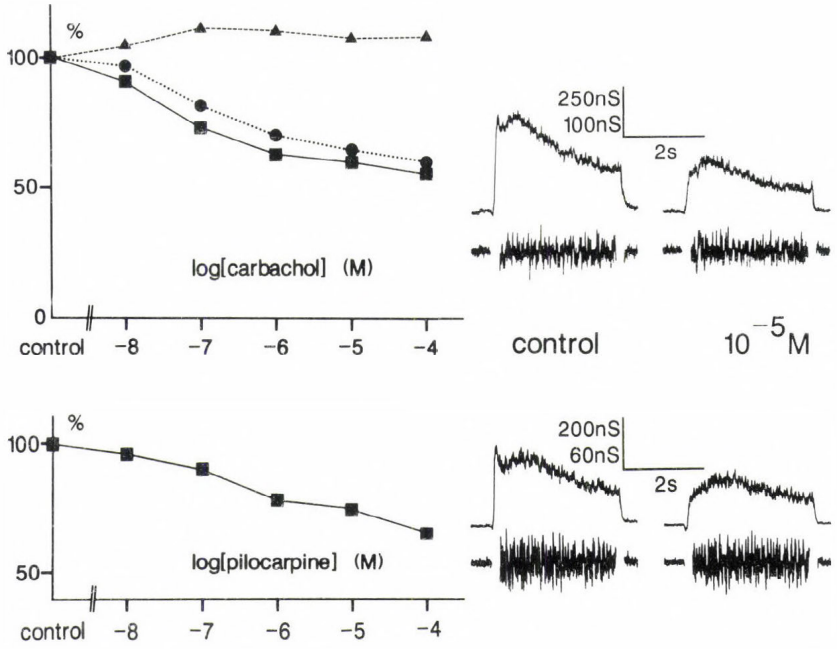

Figure 3: *Bath applied carbachol and pilocarpine decreased quantal ACh release (■).*

The number of ACh quanta released was calculated from the relative amplitudes of the long duration response, I (●) and of the MPSC (▲). On the right, recordings are the long duration responses of a postsynaptic cell voltage-clamped at -80mV induced by depolarizations of the presynaptic cell to +10mV under control conditions and after application of carbachol (10^{-5}M) or pilocarpine (10^{-5}M). Upper traces: DC recordings, lower traces: high gain AC recordings. Carbachol : 100% is 184 nS for I, 1.04 nS for MPSC, 38000 quanta per LDIPSC (T_{min}=14 ms). Pilocarpine : 100% is 37600 quanta per LDIPSC (T_{min}=13.5 ms).

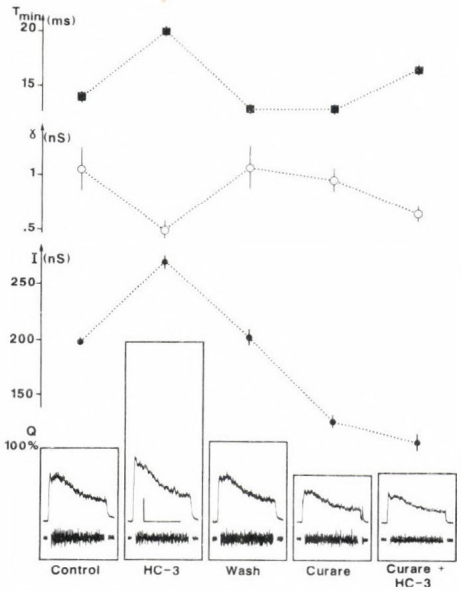

Figure 4: *Curare (3.10^{-6}M) prevented the facilitatory action of HC-3 (3.10^{-6}M) on the quantal ACh release.*

*At the bottom, long duration postsynaptic responses are shown in d.c. (upper trace) and high gain a.c. (lower trace) recordings. Vertical calibrations inside the second block are 150 nS for d.c. recordings and 60 nS for a.c. recordings. Horizontal calibration is 2 s. Long duration postsynaptic responses were induced by a depolarization to +10 mV of the presynaptic cell for 3 seconds. The blocks in which responses are inserted represent the number of quanta released per stimulus calculated from postsynaptic response amplitude, I (●) and MPSC amplitude, (○) and time constant, T_{min} (■) represented on the above graphs. Each value was calculated after 30 minutes of superfusion with each medium (except for wash: 60 minutes) and was the average (SD, vertical bars) from at least 6 recordings in the same experiment. 100 % for = 40,795. The facilitation of quantal ACh release by HC-3 was reversed by one hour washing. Bath application of curare induced a decrease of the quantal content (**Q**) due to its action on presynaptic nicotinic receptors. A subsequent addition of HC-3 (last block) was without effect (from Poulain et al. 1987).*

Hemicholinium-3 (HC-3), which acts as a postsynaptic cholinergic agonist, facilitates ACh release in our preparation (Baux et al. 1986). This was an unexpected finding since the main effect attributed to HC-3 is the inhibition of high affinity choline transport into the terminal thus depressing ACh synthesis and release (Birks and MacIntosh 1961). Since the potentiation of ACh release by HC-3 was prevented by curare (Figs 4 and 5) and hexamethonium but not by atropine (Fig. 5) we concluded that HC-3 activated presynaptic nicotinic-like ACh receptors (Poulain et al. 1987).

Moreover, some ACh agonists activated both muscarinic-like and nicotinic-like presynaptic receptors. For instance, oxotremorine, known as a muscarinic agonist in vertebrates, decreased the ACh release when nicotinic-like receptors were blocked by curare whereas it potentiated ACh release after the blockade of muscarinic receptors by atropine (Fig. 5).

DISCUSSION

We have referred to presynaptic receptors as nicotinic-like or muscarinic-like according to the action of postsynaptic antagonists such as curare and atropine (Baux and Tauc 1987). However, they cannot easily be compared to Aplysia postsynaptic ACh receptors.

Presynaptic nicotinic-like ACh receptors appear to be sensitive to curare and hexamethonium, as are the D-type postsynaptic receptors associated with cationic channels (Tauc and Gerschenfeld 1962). The H-type postsynaptic ACh receptors associated with chloride channels, which are the postsynaptic ACh receptors present on the synapse studied here, are blocked by curare but are insensitive to hexamethonium. On the contrary, similar to H-type postsynaptic ACh receptors, the presynaptic nicotinic-like receptors are activated by HC-3 which was without effect on D-type postsynaptic receptors.

Postsynaptic muscarinic ACh receptors have not been described in Aplysia ganglia, although atropine acts as a weak antagonist at both H- and D-type ACh receptors. However, the presence of muscarinic ACh receptor in Aplysia ganglia has been supported by a study using the stereoselective binding of ^{3}H-quinuclidinyl benzylate, QNB (Murray et al. 1985), which is widely used

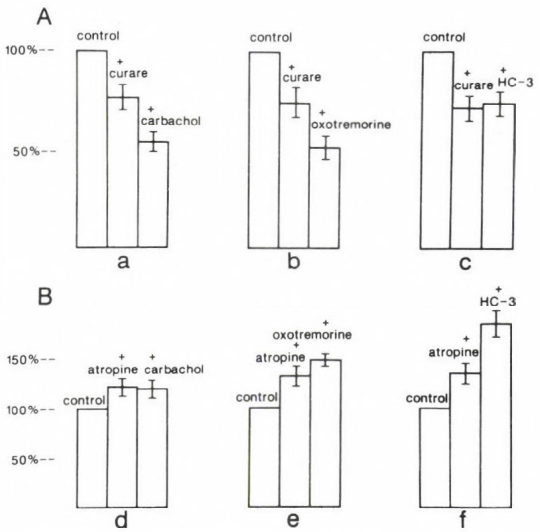

Figure 5: *Actions of cholinergic agonists, carbachol (10^{-5}M), oxotremorine (10^{-5}M) and hemicholinium-3 (3.10^{-6}M) on quantal ACh release (**Q**) after preliminary addition of curare 10^{-5}M (A) or atropine 5.10^{-6}M (B).*

*In A: All drugs except HC-3 led to a further decrease in the number of quanta released per stimulus (**Q**). In B: Atropine, contrary to curare, increased quantal ACh release (**Q**); only HC-3 and, to a lesser extent, oxotremorine induced an additional increase in quantal ACh release. Histograms represent means of different experiments done in identical conditions. Number of experiments: a=2, b=7, c=3, d=2, e=4, f=3. Vertical bars give standard deviations.*

in vertebrates as an inhibitor of the postsynaptic muscarinic ACh receptor. However, QNB was without effect on quantal ACh release on the synapse examined here. This lack of action of QNB at the presynaptic level can be explained, as was proposed for central cholinergic neurons (Szerb 1978), by specific pharmacological properties of presynaptic muscarinic receptors.

In conclusion, the experimental results show the existence of both presynaptic nicotinic-like and muscarinic-like ACh receptors on an identified cholinergic terminal. The demonstration of the presence of both types of receptors, already proposed for synaptosomes of the myenteric plexus of guinea pig (Briggs and Cooper 1982) is the first evidence for such a regulatory mechanism in a neuro-neuronal synapse in invertebrates. Moreover, these presynaptic receptors are probably autoreceptors since similar modulation was observed when ACh release was evoked either by presynaptic action potentials or presynaptic depolarizations in the presence of tetrodotoxin, which supresses the activity of other synapses.

Acknowledgements: We thank Dr John Randle for his helpful comments. The work was supported by grants 85/6021 from Institut National de la Santé et de la Recherche Médicale and 86/1197 from Direction des Recherches Etudes et Techniques to L.T.

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CATECHOLAMINES AND CHEMICALLY RELATED AMINO ACIDS IN COELENTERATES

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The first nervous system was probably established in planula-like ancestors to coelenterates and the coelenterate nervous system is generally regarded as the most simple and primordial in the animal kingdom. Nevertheless, the structurally simple coelenterate nerve net may possess surprisingly complex physiological properties and allow a rich behavioural repertoire (Pantin 1952). This may have several explanations of which some may be of general neurobiological interest.

In pelagic medusae and siphonophores, the nerve net has condensations into nerve tracts and even giant fibers for rapid conduction (Mackie 1973, Roberts and Mackie 1980). In addition, epithelial conduction implies that the functional differentiation between nervous and non-nervous tissue is less developed than in higher animals (Mackie 1965). However, the presence of chemical synapses has been indicated in both ultrastructural (Westfall 1973) and physiological (Roberts and Mackie 1980) investigations, and it is probably at the level of chemical transmission that most of the complexity of coelenterate behaviour is generated. This is most clearly demonstrated in the well known freshwater polyp Hydra, in which both co-localization of neuropeptides as well as chemically distinct peptidergic nerve-plexa have been demonstrated (Grimmelikhuijzen 1983a).

Despite the probable key role of different neurotransmitters in extending the functional possibilities of a topographically simple nervous system, the knowledge about these compounds is sparse and contradictory (Martin and Spencer 1983). So far,

the only substance that has been satisfactorily characterized as neurotransmitter in coelenterates is antho-RFamide (Grimmelikhuijzen and Graff 1986) and this peptide appears to be widely distributed among coelenterates (Grimmelikhuijzen 1983b). Whereas peptidergic neurotransmission seems to be well established in coelenterates, the role of "traditional" neurotransmitters, e.g. acetylcholine, GABA, glycine, glutamate and biogenic amines, is still uncertain. This work will touch some of the problems associated with the last mentioned group: the biogenic monoamines.

MONOAMINERGIC NEUROTRANSMISSION

Monoaminergic neurotransmission is well developed in the mammalian nervous system in which dopaminergic, noradrenergic, adrenergic and serotoninergic nerve tracts form a complex network. Even in invertebrates, monoaminergic nerves have been established at least down to the planarians (Welsh and Williams 1970). In coelenterates, monoaminergic neurotransmission is less certain, but evidences obtained by different methods are slowly accumulating.

Histochemical evidences

The condensation of catecholamines with dry formaldehyde vapour into fluorescent isoquinolines (Falck and Hillarp 1962) has been extensively used for mapping monoaminergic neurons in mammalian nervous systems. The reaction mechanism is well understood (Corrodi and Jonsson 1967) and selective for catecholamines and related amino acids (Fig. 1). This technique has provided histochemical evidences for a monoaminergic nervous system in the tentacles of sea anemones (Dahl et al. 1963) and in the ectoderm of Hydra (Castano and Rossi 1978). Based on a chemically similar principle, the glyoxylic acid method of De la Torre (1980) has been applied on whole-mounts of the ctenophore Mnemiopsis leidyi, and there revealed fluorescent cells in the proximal part of the meridional canals (Carlberg, in

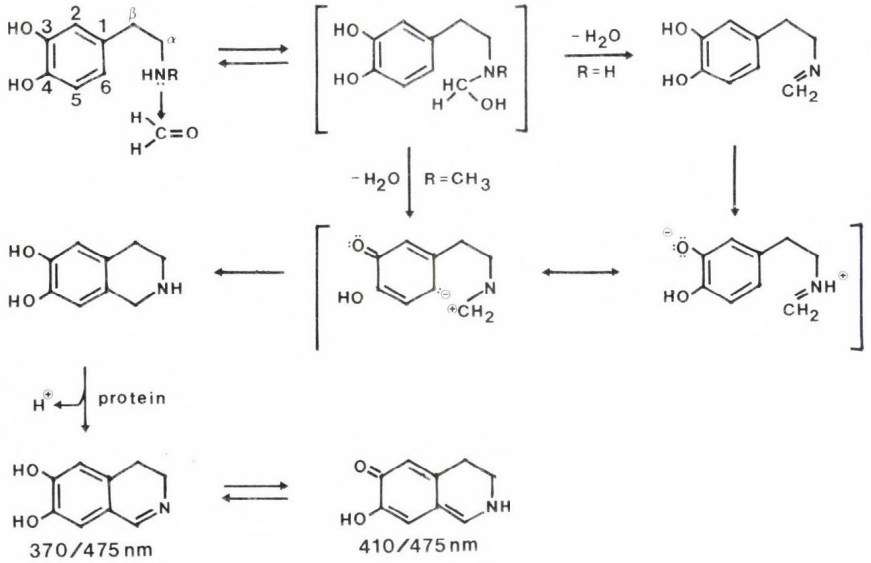

Fig. 1. Reaction mechanisms in the Falck-Hillarp method. The amino group of the aliphatic side chain reacts by nucleophilic addition to the carbon atom in formaldehyde. The reaction is followed by cyclization and protein-catalyzed reduction into fluorescent isoquinolines. Ring-closure is dependent on an activating hydroxy-group at 3-position. The isoquinolines have excitation maxima around 370-410 nm and emit light of 475 nm wavelength. Fluorescent structures are studied in a UV-microscope with appropriate filter settings.

press). However, by the methods used it is not possible to distinguish between the reaction products of different catecholamines.

Pharmacological evidences

Ancil et al. (1982) report that adrenaline has direct effects on bioluminescence and modulatory effects on contractility in the sea pansy Renilla köllikeri. The effect of adrenaline was counteracted by propranolol (1mM), 6-OH-dopamine and reserpine, whereas desmethylimipramine (20 µM) and pargyline potentiated the effects of adrenaline. The results suggest similarities to vertebrate beta-adrenergic systems. A similar

adrenergic system may also control the bioluminescence in the ctenophore Mnemiopsis leidyi (Anctil 1985).

Analytic-chemical evidences

Thin-layer chromatography has occasionally been employed to show 2,3-dihydroxyphenylalanine (DOPA), dopamine and noradrenaline in sea anemones (Carlyle 1969, Lenique et al. 1977). The access to high performance liquid chromatography (HPLC) with electrochemical detection has greatly extended the possibilities for analysing coelenterate tissues on endogenous catecholamines. An HPLC-analysis of 20 different coelenterate species including Anthozoans, Scyphozoans, Hydrozoans and ctenophores has increased the knowledge about the distribution of catecholamines and related amino acids in coelenterates (Carlberg and Rosengren 1985). Accordingly, dopamine was found in several species and noradrenaline was recovered in the athecate hydrozoans Hydra and Tubularia. 5-HT was detected in the syphozoan Cyanea lamarcki, but adrenaline was not demonstrable in any of the species examined. However, adrenaline may possibly occur in the ctenophore Mnemiopsis leidyi (Carlberg, in press) and in the sea pansy Renilla köllikeri (De Waele et al., in press). Another interesting property of coelenterates is their content of large amounts of DOPA and several species also contain DOPA-metabolites formed through the action of tyrosinase,* e.g. 5-S-cysteinyl DOPA and 3,4,5-trihydroxyphenylalanine (5-OH-DOPA). Since coelenterate nervous elements are virtually impossible to dissect out, analytic-chemical methods do not provide any information about the localization of different catechol compounds.

THE DOPA/5-OH-DOPA-SYSTEM

As discovered by Dahl et al. (1963), the tentacles of sea anemones contain epithelial sensory cells and an interconnected

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* o-diphenol: O_2 oxidoreductase

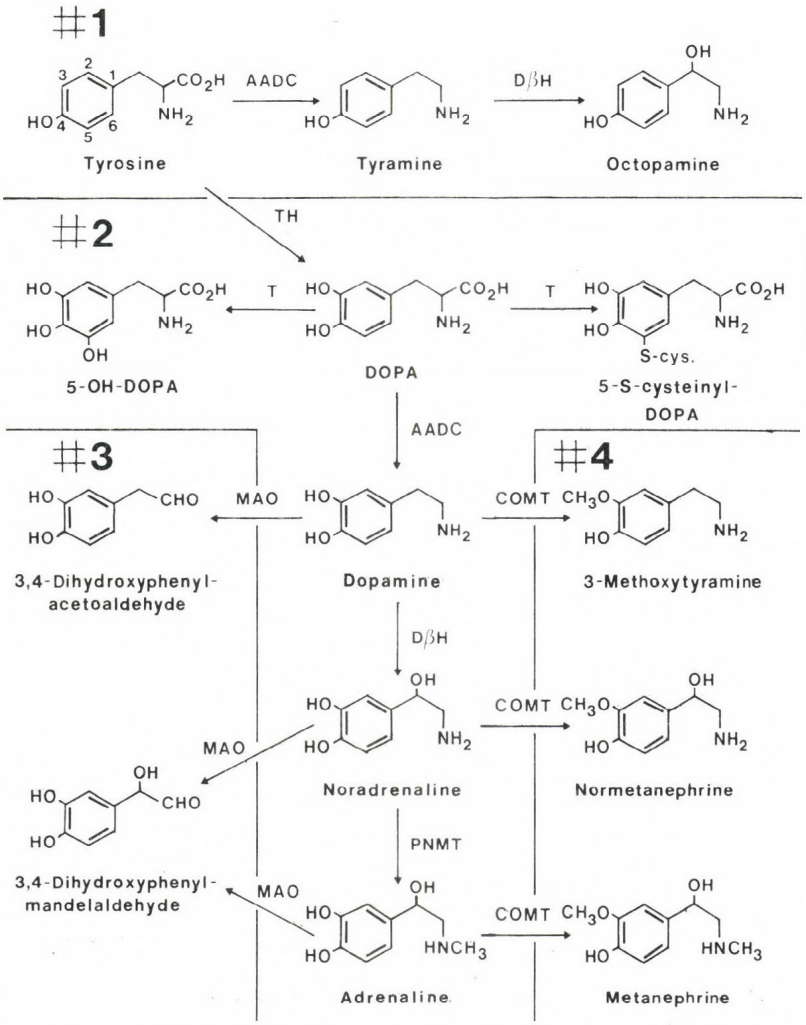

Fig. 2. Possible pathways in the biosynthesis and metabolism of DOPA and catecholamines. Compounds that fulfil the molecular requirements for forming fluorescent reaction products with formaldehyde are enclosed in the T-shaped area #2. In monoaminergic nerves of vertebrates, the slow biosynthesis of DOPA by tyrosine hydroxylase and its rapid decarboxylation in the cystosol, generally does not allow steady state levels of DOPA high enough to give any significant contribution to the histofluorescence seen in those cells. Compounds within area #1 and 4 do not possess the hydroxy-group at 3-position and the compounds within area #3 lack the aliphatic amine group that reacts with formaldehyde. Abbreviations: AADC: aromatic amino acid decarboxylase, DβH: dopamine-β-hydroxylase, TH: tyrosine hydroxylase, T: tyrosinase, COMT: catechol-o-methyltransferase, MAO: monoamine oxidase, PNMT: phenylethylamine-N-methyltransferase.

subepithelial nerve plexus that both fluoresce when treated with the Falck-Hillarp method for visualising monoaminergic nerves. However, an HPLC-analysis of tentacles from the sea anemones Tealia felina and Metridium senile showed high amounts of DOPA concentrated to tentacular tissue (Carlberg 1983). Among possible DOPA-metabolites, 5-OH-DOPA and cysteinyl DOPAs were recovered, whereas catecholamines were not detectable. All these substances fulfil the molecular requirements for forming fluorescent reaction products with formaldehyde (Fig. 2).

The identity of 5-OH-DOPA has been verified by gas chromatography-mass spectometry (GC-MS) (Carlberg et al. 1982) and it has been found to be formed through a tyrosinase-mediated mixed-function oxidase reaction in which ascorbic acid may act as hydrogen-donating cofactor (Carlberg et al. 1984). DOPA and 5-OH-DOPA are probably formed inside subcellular vesicles that have been enriched on sucrose density gradients and recovered in situ in epithelial sensory cells and in the ectodermal base-pithelial nerve plexus in the tentacles of the sea anemone Metridium senile (Carlberg and Elofsson, in press). The formaldehyde-induced histofluorescence seen in tentacles of sea anemones is thus most probably explained by a tyrosinase-mediated accumulation of DOPA and 5-OH-DOPA.

The glyoxylic acid-induced histofluorescence seen in the meridional canals of the ctenophore Mnemiopsis is most probably caused by DOPA and/or 5-S-cysteinyl DOPA (Carlberg, in press), indicating the action of a tyrosinase. Since both compounds were found in small amounts, it is possible that 5-OH-DOPA fell below the detection limit. However, 5-OH-DOPA has been found in several other ctenophoran species (Carlberg and Rosengren 1985). The fluorescent cells in Mnemiopsis do not show any mark characteristics of nerve cells, but accessory situated cells containing dense core vesicles may suggest that they are innervated and their position in the canal wall may imply some endocrine function (Carlberg, in press).

CONCLUSIONS

Among possible transmitter candidates, 5-OH-DOPA has some interesting chemical properties. At the extracellular pH of sea anemones as well as other marine poikilosmotic invertebrates (~8.2), it is rapidly oxidized ($t_{1/2}$~1.5 min, unpublished results) into stable, non-polymerizable (Agrup et al. 1982), diffusible and probably non-toxic compounds. Considering the tyrosinase-dependence of 5-OH-DOPA and the fact that the safer pteridine-dependent tyrosine hydroxylase system has taken over the DOPA-biosynthesis already in the nervous systems of crustaceans (Laxmyr 1985), 5-OH-DOPA as a neurotransmitter is probably a phenomenon restricted to lower invertebrates.

However, the DOPA/5-OH-DOPA-system may be widely distributed among coelenterates, and in several species it may even coexist with catecholamines. Since the histofluorescence methods based on aldehyde condensation into isoquinolines do not allow any distinction between different catechol compounds, nothing is ascertained about the localization and spatial relationship between monoaminergic and DOPA/5-OH-DOPA-containing cells in coelenterate species. Advances in the techniques for raising antibodies against low-molecular compounds will probably soon make it possible to perform immunohistochemical localization of different catechol compounds in coelenterates. Less certain is if the biochemical and physiological responses that the different catechols may elicit are within reach of available techniques and human imagination. Since the coelenterate nerves do not attract many investigators, it is questionable if even the most simple nervous systems ever will be completely understood.

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DISCUSSION

ELEKES, K.: Did you find other types of nerve terminals or axon profiles in the nerve plexus with other type(s) of granules?

What about FMRF-amide containing elements?

CARLBERG, M.: In addition, there are profiles containing opaque vesicles. These profiles show immunoreactivity to FMRF-amide and they constitute a network that is separate from the intermingled catechol-reactive processes originating from epithelial sensory cells.

WALKER, R.J.: Is there any evidence that 5-OH-DOPA has any biological activity? Normally DOPA has no effect until it is decarboxylated.

CARLBERG, M.: The biological activity of 5-OH-DOPA is still unknown. First, it has to be established in cells that are suitable for physiological experiments. Attempts to study the effects of 5-OH-DOPA and L-DOPA on adenylcyclase have been hampered by an enormously high basal activity of this enzyme in membranes from Metridium-tentacles. Possibly, the adenylcyclase is regulated by some diffusible inhibitory factor (maybe a G_i-protein). Radiolabelled L-DOPA has also been used in receptor binding assays, but so far all binding has been dismissed as covalent binding of oxidation products to thiol-groups of membrane proteins. If there are receptors for L-DOPA or 5-OH-DOPA, a main problem is that the K_D has to be low enough to permit washing of the membranes without loss of bound radiolabelled ligand through diffusion. On the other hand, the K_D has to be high enough to permit the binding of measurable amounts of radiolabelled ligand when the ligand is present in concentrations close to the K_D.

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THE LEECH RETZIUS CELL: A MULTIACTION NEURONE

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INTRODUCTION

The precise role of 5-hydroxytryptamine (5-HT: serotonin) in the nervous system is not yet known although there are large numbers of serotoninergic neurones in the mammalian brain. The leech, however, possesses less than 250 5-HT-containing neurones in its entire nervous system and these are apportioned as a small population in each ganglion of the ventral nerve cord. Thus, it is perhaps simpler to study the possible role(s) of 5-HT in such an animal.

Although the leech nervous system consists of 32 segmental ganglia, the most anterior 4 and posterior 7 of which are fused to form "brains" associated with the suckers, the 21 free segmental ganglia (G1-21) are similar but not identical. Equally the distribution of 5-HT-containing neurones is not identical in each ganglion and also varies a little in different species. In the medicinal leech, *Hirudo medicinalis*, all ganglia possess a pair each of giant Retzius (R) cells, dorso-lateral (cell 21) and ventro-lateral (cell 61) serotoninergic neurones as well as a normally (but not invariably) unpaired median (M) cell. In addition, G1-3 contain an extra (E) pair of cells while the anterior brain possesses, in the first segment, a single pair of large lateral (LL) neurones which send processes into the head (Lent, 1985a). All the other 5-HT-containing cells confine

their connections to the central nervous system, with the exception of the R cells.

Each R cell sends all its processes ipsilaterally, with major branches in the paired lateral roots and smaller branches in the anterior and posterior connectives, as well as an extensive dendritic tree in the neuropile (Mason & Leake, 1978). It contacts peripheral tissues via the segmental nerves of its own and the adjacent two ganglia. All the peripheral tissues so far investigated contain nerves with 5-HT-like immunoreactivity but none possesses any serotoninergic cell bodies except for the crop region of the gut which has a scattering of 5-HT-like immunoreactive neurones (Leake et al., 1986). Thus, although it has not been possible to trace the finer branches of the R cells, it would seem likely that all peripheral tissues are innervated by them.

Some years ago it was shown that the R cells could control mucus release from the skin, an action that could be mimicked by 5-HT (Lent, 1973), and it was assumed that this was their only function. However, more recent evidence suggests that they can mediate a whole range of actions. For instance, R cell stimulation and 5-HT application both decrease resting tension in the longitudinal and circular muscles of the body wall and increase their rate of relaxation following contractions induced by either direct electrical stimulation or stimulation of their excitatory motoneurones (Mason et al., 1979 ; Leake et al., 1981 ; Mason & Kristan, 1982). 5-HT also affects all the other tissues of the body which receive a serotoninergic innervation. It enhances pharyngeal contractions and salivary gland secretion at 10^{-8}-10^{-6}M as well as increasing biting frequency. Stimulation of the R cells mimicks the action on the salivary gland cells whereas the LL cells induce movements of the pharynx (Lent & Dickinson, 1984a). 5-HT (10^{-6}-10^{-5}M) also inhibits contractions of the vagina and penis, although they are enhanced by lower concentrations ; reduces resting tension of the hind end of the gut and suppresses contractions induced by electrical stimulation ; and

potentiates contractions of the dorso-ventral muscles (Leake, 1986).

All these observations support the suggestion that the role of 5-HT, as produced by the R cells, is to organise optimal feeding efficiency in the leech (Lent, 1985a ; Leake, 1986). The R cells themselves are segmentally iterated but their target tissues are not. Thus, perhaps, one can assume that the R cells do not all behave in the same way at any one time. In this paper we investigate some of this behaviour of R cells in hungry and recently fed leeches.

MATERIALS AND METHODS

Leeches (*Hirudo medicinalis*) were obtained from Biopharm (U.K.) Ltd, Swansea and were kept in 4% leech Ringer at room temperature (15-20°C). Hungry leeches had not fed for at least 3 months but several still showed traces of blood in the gut. Therefore, some were forced to regurgitate any remaining traces of food by being immersed in 6% saline for a few minutes at least 4 days before the experiment. For feeding, leeches were lowered into fresh, warm, heparinised (10mg l^{-1}) pig blood in a muslin bag. Only 'good' feeders were used, i.e. those individuals which started to pump in blood immediately and did not stop until they were sated, by which time they were 8-10 times their previous size. Fed leeches were then washed in 4% Ringer.

For intracellular recording one anterior (G4), one mid-body (G12) and one posterior (G18) ganglion were removed from each leech in random order, each ganglion remaining *in situ* until just before the experiment. Each isolated ganglion was mounted on a Sylgard block in the experimental bath and intracellular recordings were made using capillary-inset glass microelectrodes filled with 4M KCl (R = 25-40 MΩ). Accurate measurements were made of resting potential (RP), height and duration of action potential (AP) and after hyperpolarisation (AHP) directly off the screen of a Tektronix 5113N storage oscilloscope, and of AP frequency from traces obtained on a Gould Brush 2400 pen recorder. Several minutes were allowed for equilibration after impaling an R

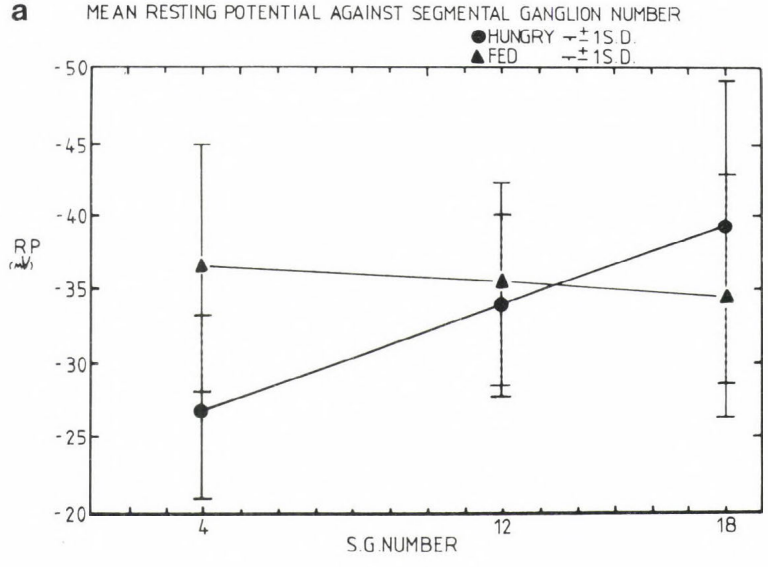
cell before measurements were made. One hungry and one fed leech were used each day; fed leeches being used up to 10 days after feeding.

For fluorescence histochemistry whole nerve cords were excised from hungry and fed leeches (selected at random) and dropped quickly into ice-cold leech Ringer. They were incubated at 4°C for 1h in a sucrose, potassium phosphate, glyoxylic acid (SPG) solution (Lent, 1982), dried in a stream of cool air, cooked at 90°C for 3 minutes, mounted in liquid paraffin and viewed under UV light using a Leitz Dialux 22EB microscope.

RESULTS

Intracellular recording

Measurements of parameters of R cell activity in hungry and fed leeches (expressed as means of all animals tested before and after feeding) are shown in Table 1. In hungry leeches the only parameter that differed between ganglia was the RP which was significantly smaller in R cells in G4 than in G12 or G18 (Fig. 1a). This difference between ganglia



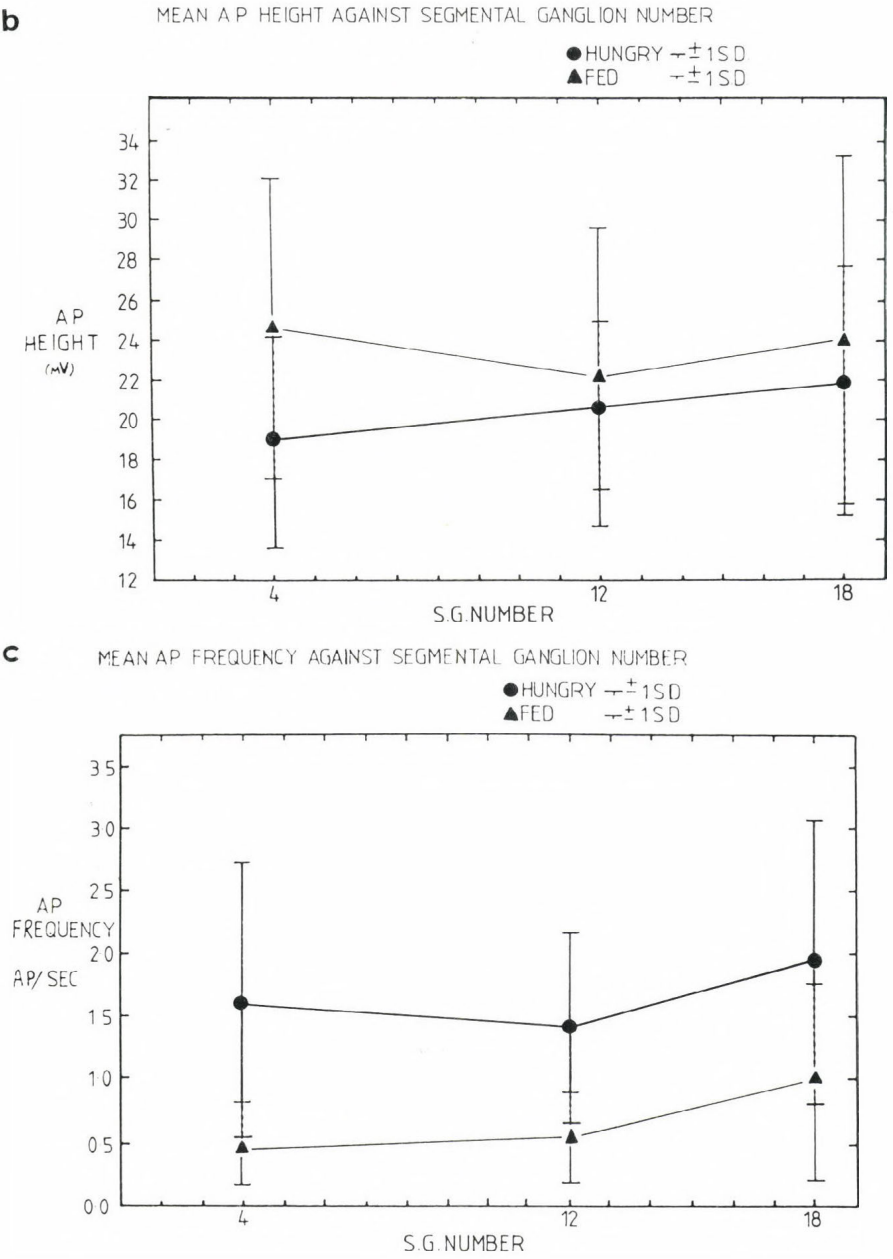


Fig.1 Mean values of (a) RP, (b) AP height and (c) AP frequency in R cells of segmental ganglia 4, 12 and 18 in hungry (●) and fed (▲) leeches.

Table 1 Comparison of mean ($\bar{x}$) measurements of R cell activity in segmental ganglia G4, G12 and G18 in hungry (H) and fed (F) leeches.

		G4			4;12	G12			12;18	G18			4;18
		n	$\bar{x}$	S.D.		n	$\bar{x}$	S.D.		n	$\bar{x}$	S.D.	
RP (mV)	H	17	-27.0	6.2	**	13	-34.2	6.6		18	-39.1	10.5	**
	F	22	-36.6	8.6		22	-35.4	6.9		22	-34.8	8.3	
	H;F		**										
AP height (mV)	H	18	19.0	5.3		14	20.8	4.5		18	21.8	6.0	
	F	22	24.7	7.5		22	22.2	7.4		22	24.2	9.0	
	H;F		**										
AP duration (ms)	H	18	7.9	2.0		14	7.4	2.1		18	7.1	1.7	
	F	22	7.8	3.3		22	6.8	2.4		22	7.3	2.3	
	H;F												
AP frequency (Hz)	H	15	1.6	1.1		14	1.4	0.8	**	17	2.0	1.1	
	F	21	0.5	0.3		20	0.6	0.4	**	22	1.0	0.8	
	H;F		**				**						
AHP height (mV)	H	18	4.0	2.2		14	5.0	3.0		18	5.0	1.8	
	F	22	5.3	2.2		22	5.8	2.1		22	5.8	1.9	
	H;F		*										
AHP duration (ms)	H	18	91.8	14.5		14	102.0	20.3		18	97.6	25.3	
	F	22	94.8	18.7		22	91.3	18.9		22	106.6	20.1	
	H;F										**		

*, significant at $P < 0.05$; **, significant at $P < 0.01$,
n = no. of samples; S.D. = standard deviation

disappeared in fed leeches, when the mean RP of R cells in G4 increased significantly and became indistinguishable from those in G12 and G18. The increase in size of RP in R cells of G4 after feeding was accompanied by a significant decrease in the mean frequency and an increase in the mean size of APs (Fig. 1b,c). A decrease in AP rate was also seen in R cells of G12 and G18 after feeding but the small increases in AP amplitude seen were not significant. Mean height of AHP also increased slightly after feeding in all ganglia but was only significant at the 5% level in R cells of G4.

However, all the fed animals were treated as one population in these comparisons. If the mean values for animals sacrificed at different times after feeding were taken (Fig. 2a), then the RP values of R cells in all three ganglia were seen to increase for the first 6 days after feeding, before a secondary drop in value.

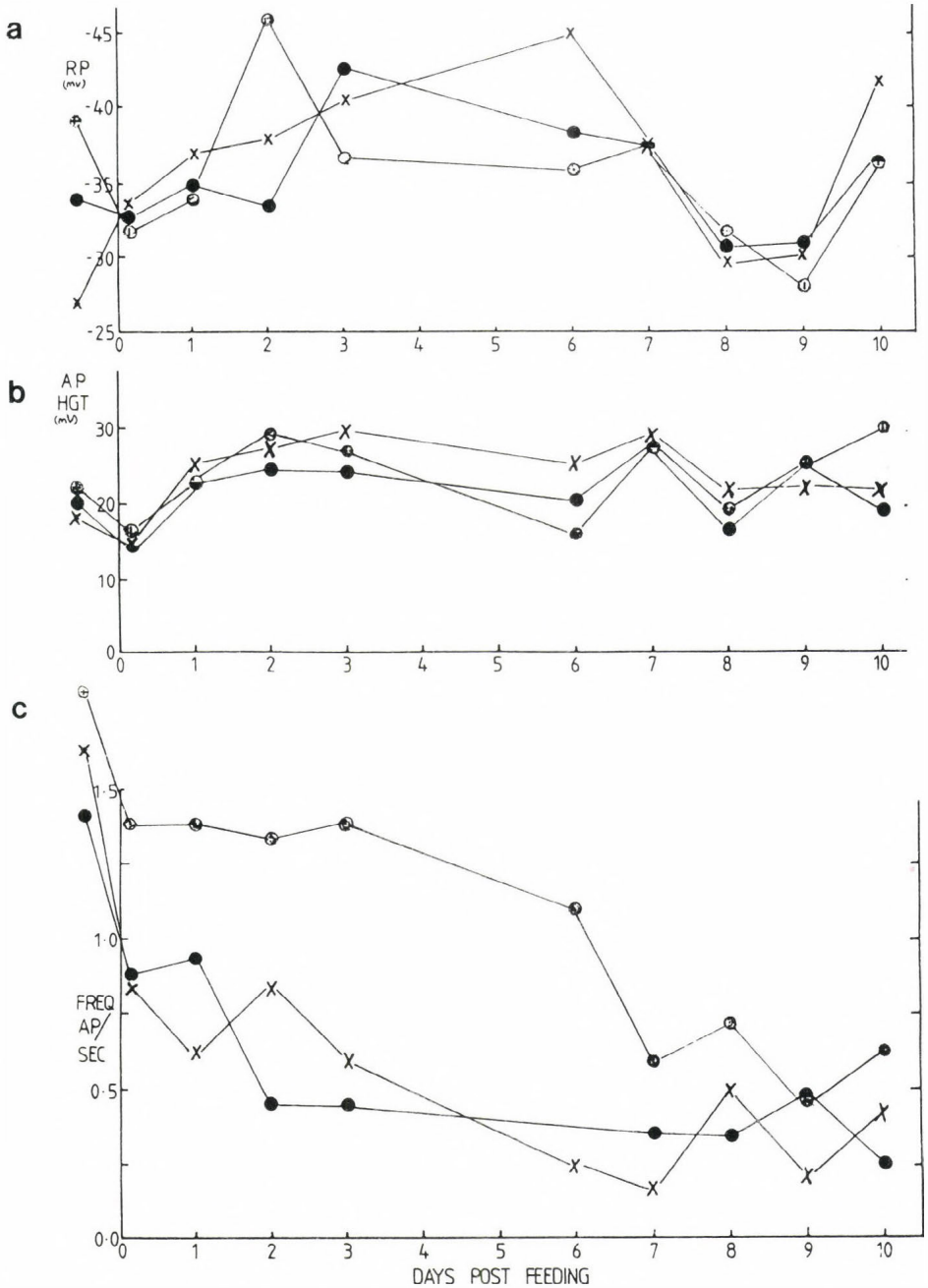


Fig.2 Mean values of (a) RP, (b) AP height and (c) AP frequency in R cells of individual ganglia (X = G4, ● = G12 , ⊕ = G18) before (n = 18) and days after (n = 1-3) feeding.

On the other hand, increase in AP height remained constant for at least 10 days after feeding while frequency of APs declined in a steady manner (Fig.2b,c). The mean frequency of APs in R cells in G18, however, remained substantially higher than those in G4 and G12 until day 7 after feeding.

Fluorescence histochemistry

All ganglia of the nerve cords showed fluorescence staining of a small number of neurones. Under all treatments the DL and VL cells behaved in the same way and showed bright fluorescence, while that of the M cell was very variable. On the other hand, the Retzius cells showed a level of fluorescence related to diet. Nerve cords of hungry leeches which had been forced to regurgitate possessed brightly fluorescent cells throughout the nerve cord (Fig.3a). In leeches which were hungry but whose guts still contained traces of food, the R cells showed more variable fluorescence although the cells in G5, G6, G20, G21, sub-oesophageal and caudal ganglia were always bright. R cells in G1-4 and G7-19 showed lower levels of fluorescence and the two (right and left) cells in any one ganglion did not necessarily show the same level. This unequal distribution of fluorescence could also occur in fed leeches. Immediately (<4h) after feeding, all ganglia, except G5, G6, G20 and G21 in which fluorescence levels were medium-to-bright, showed no fluorescence in both R cells or dim fluorescence in one R cell and none in the other (Fig.3b,c). By 24h after feeding, fluorescence levels in R cells had increased but one of the paired cells still often showed no fluorescence. A number of brown blebs were often seen inside the cell, usually associated with the large nucleus (Fig.3d).

DISCUSSION

Results from both sets of experiments support the idea that all the R cells within an individual leech do not necessarily behave in the same way at any one time.

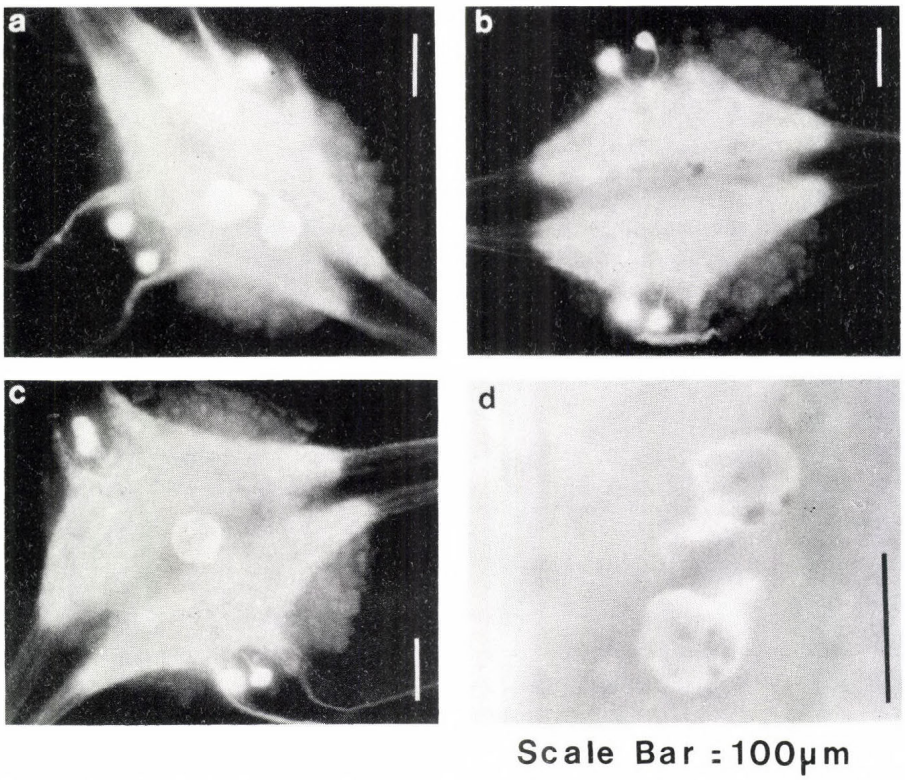


Fig.3. SPG fluorescence in 5-HT-containing cells in mid-body ganglia of the leech; note two lateral DL and VL cells (sometimes superimposed) and single posterior M cell. The large cells are R cells. (a) Hungry leech: note two very bright R cells. (b) Fed leech after 24h; R cells only visible as brown blebs. (c) Fed leech after 5 days: note single R cell, the other is not visible. (d) Fed leech after 4 days: R cells showing brown blebs.

In the hungry leeches, although all the R cells appeared to contain similar amounts of 5-HT, as judged by SPG fluorescence, there is clearly a gradient of mean resting potential down the animal with the R cells of G4 being the least negative. This implies that the more anterior R cells are nearer to the firing threshold in hungry leeches, although this threshold has not been exceeded since the

actual rate of APs is not faster than in R cells in the more posterior ganglia. However, it can be inferred that the anterior R cells are likely to be fired more readily to release their 5-HT in hungry animals. The R cells of G4 are closest to the structures most directly involved with feeding, i.e. the jaws, salivary glands and pharynx (Lent & Dickinson, 1984a) and are likely to switch on these structures to produce the fixed action pattern of feeding. However, it would seem from the fluorescence study that all the R cells are full of 5-HT in hungry leeches, so presumably it can be released along the length of the leech to integrate the whole behaviour pattern. The SPG method is not strictly quantitative but Lent & Dickinson (1984a) have shown there is a relationship between level of fluorescence and 5-HT content, although it is not known what concentrations of 5-HT the method can distinguish.

Immediately the leeches have been fed they are behaviourally depressed, i.e. they cease to feed, do not respond to stimuli and are inactive with the distended body supported by the water below the surface in their container. This behaviour fits in with the observation that the mean RP values of the R cells in G4 become hyperpolarised and the firing frequency falls dramatically, although the APs are actually larger when they do fire. The gradient of RP values along the length of the leech disappears (see Fig.1a). However, these values are from pooled data. If values taken on separate days after feeding are used (see Fig.2a), more detail is revealed, i.e. the RP values increase (hyper-polarise) steadily up to 6 days post feeding and then fall for 3 days before rising again. Unfortunately, because of the small number (1-3) of animals used on any particular day, one cannot test for significance. Similarly, whereas the pooled data on AP frequency (Fig.1b) reveals a significant drop in firing rate after feeding, the individual data points (Fig.2b) reveal that, although R cells in G18 fire slightly faster in hungry leeches, in fed animals they fire at about twice the rate of those in G4 and G12 until day 6 when they drop to similar rates. This could suggest a separate role

for R cells at the hind end of the leech after feeding that is not apparent before feeding. It is possible that in the hind end of the animal continuing 'shots' of 5-HT are required at intervals after feeding to keep the gut and body wall muscles distended until all the stored blood has disappeared. These results also indicate that events in the R cells after feeding may go through at least one cycle lasting for 6-7 days. It is obviously important to follow activity for longer than 10 days post feeding.

The fluorescence results in turn suggest that perhaps the critical events in the R cells associated with feeding occur in the first few hours after feeding when most of the cells are depleted of fluorescent material. Lent (1985b) has shown that the mean levels of 5-HT in the ventral nerve cord fall from 13-17pmol/ganglion before feeding to 2.5pmol/ganglion after feeding with the largest decreases in the more anterior ganglia but none in the caudal ganglion. Our fluorescence results suggest, firstly, that it is depletion of 5-HT in the Retzius cells which largely accounts for this fall but, also, that the levels do not fall significantly in G20 and G21 as well as in the caudal ganglion or in the sex ganglia G5 and G6. Perhaps it can be assumed that 5-HT levels in ganglia right at the posterior end of the leech are associated with continued suppression of hindgut activity (Leake, 1986) or activation of the posterior sucker even after feeding. It is also possible that 5-HT released by R cells in G5 and G6 not only inhibits movements of the muscular sex organs in a sustained manner (Leake, 1986), but has an additional role at the gonopores which are particularly heavily innervated by the R cells (Glover & Mason, 1986).

In a number, but not all, of the fed or part-fed leeches the two R cells showed a different level or fluorescence. This difference between cells was not shown by the other serotoninergic neurones and implies that the peripherally-released 5-HT in a segment may be released sequentially by first one R cell and then its pair. It was also noticeable that in part-fed leeches the R cells in adjacent ganglia did not always show the same level of

fluorescence, also suggesting different functions for cells in different ganglia. The appearance of distinct brown blebs in R cells in leeches from about 24 hrs after feeding is puzzling. Such blebs have previously been described by Lent & Dickinson (1984b) in R cells from leeches treated with the neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), and attributed to oxidation products of 5,7-DHT. It appears from our results that the blebs may well be a natural breakdown product of 5-HT. They certainly appear to be associated with lowered levels of 5-HT.

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DISCUSSION

ELEKES, K.: You have presented a multifunctional role for the Retzius cells, innervating a series of peripheral organs and regulating several peripheral processes. Did you investigate the possible different types of connections of Retzius-cell terminals at the EM-level?

LEAKE, L.: We have looked for 5-HT containing terminals in the body wall muscles and found some but the serotonergic innervation of the leech tissues is much more sparse than in vertebrates, so the nerve endings are difficult to find.

JANSE, C.: You showed that a great number of organs are innervated by Retzius cells. Does each Retzius cell innervate all organs or is there a subdivision? In relation with this, is there a subdivision of functions between the Retzius cells?

LEAKE, L.: The branches of the R cells have not been followed out to all organs, but it is known (Glover and Mason, 1986) that the R cells in the sex ganglia (G5 and 6) divert a large number of axon processes, including those that normally run into the connectives, towards the genopores and other sexual structures. Perhaps one can assume that the R cells go to all tissues present but divert some processes to specialised tissues when they occur.

KEMENES, G.: Had the brown pigment disappeared from the Retzius cells by the time the animals became hungry again?

LEAKE, L.: Yes, in really hungry leeches there was no sign of pigment but brown blebs could still be seen several weeks after feeding.

LUKOWIAK, K.: 1. Do your 5,7 DHT treated animals still eat normally?

2. What about changes in staining intensity at terminals, are they correlated with the "state" of the leech?

LEAKE, L.: 1. We have not treated leeches with 5,7 DHT but Lent has shown that 5,7 DHT-treated animals lose their appetite.

2. We have not yet looked at staining intensity at the nerve terminals. That is something to investigate further.

NÄSSEL, D.R.: Do you know what the inputs or central connections of the Retzius cells are?

LEAKE, L.: We have searched for connections and found no monosynaptic connections. However, a number of cells, especially the nociceptive (N) cells, make polysynaptic connections into the R cells.

SALÁNKI, J.: Does 5-HT disappear from the Retzius cells during feeding as a result of release at strong muscular activity, or does it release after feeding, as a result of filling up the animal with blood?

LEAKE, L.: It appears to disappear immediately the leech feeds, presumably to co-ordinate the events associated with feeding.

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THE EFFECTS OF COCAINE ON PERIPHERAL MONOAMINERGIC REGULATORY MECHANISMS IN THE GILL OF MYTILUS EDULIS

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Recently, Aiello et al. (1986) demonstrated that various opiates can modulate the central dopaminergic (DA) inhibitory mechanism associated with regulating peripheral lateral ciliary activity. Previously, in Mytilus it was demonstrated that serotonin (5-HT) applied to either the cerebral ganglion (Aiello et al., 1986), visceral ganglion or gill would cause cilioexcitation. Dopamine applied to the same tissues would cause cilioinhibition (Aiello et al., 1986), thus establishing an antagonistic system in the regulation of lateral ciliary activity. In subsequent studies these signal molecules were found to be endogenous to these tissues. These observations led to the conclusion that the visceral ganglion supplies dopaminergic, cilioinhibitory nerves to the lateral epithelium of the gill. A parallel series of observations indicates that the visceral ganglion also supplies the gill with a serotonergic, cilioexcitatory innervation. The recent report of Aiello et al. (1986) incorporates a physiological role for endogenous opioids in regulating DA activity. Briefly, DA applied to the cerebral ganglion inhibited lateral ciliary activity. This effect can be blocked by applying opioids to the visceral ganglion, which is situated in the cerebral-gill neural pathway. This blockade of dopamine cilioinhibition by opiates can be reversed by the opiate antagonist naloxone. In preparations with low endogenous rates of ciliary beating, the beating rate was stimulated by opiates, an effect reversed by naloxone. Preparations with high endogenous rates were inhibited by naloxone addition to the visceral ganglion. From these studies and others noted in that

report, it was postulated that the cilioinhibitory DA mechanism includes nerves running from the cerebral ganglion to the gill with synaptic transmission in the visceral ganglion that can be modulated by endogenous opioids. The mechanisms operating are believed to be the presynaptic inhibition of DA release by opioids. Earlier, opiates were shown to inhibit DA release in this ganglion (Leung and Stefano, 1987). Thus opiates applied to the visceral ganglion would stop dopaminergic release, effectively blocking the cilioinhibitory mechanism. Therefore, the cilioexcitation caused by opiates would be due to unmasking or allowing the 5-HT system to operate freely. In the present study we examine the effect of cocaine, a strong substance of abuse, to determine if this compound also can effect this neural mechanism.

MATERIAL AND METHODS

Specimens of subtidal Mytilus edulis 2-4 cm long were collected from Long Island Sound at Northport, New York. Healthy animals were identified as those which close when the mantle was touched. The animals were used within an 8-hour period from the time they were harvested.

Dopamine Release Assay

Gills are incubated at 22^{o}C for 30 minutes in 1 ml of ASW containing 0.1 percent ascorbic acid and [^{3}H] dopamine or serotonin (0.7 x 10 dis/min; specific activity, 35.6 Ci/mmole) (New England Nuclear) with constant shaking. After being incubated, the gills are washed twice in 2 ml of ASW and then each gill is cut into pieces (average wet weight 65 mg) and is transferred to a Plexiglass perfusion chamber containing 2 ml of ASW. An average of 6500 cpm is present in the gill at the start of the superfusion. A four-channel peristaltic pump (Rainin) maintained the flow rate at 1 ml/5 min to an inflow opening at the bottom of the chamber. The perfusing solution is altered at the desired intervals by manually transporting the

inflow tubes to the appropriate beaker. The superfusate is collected from an outflow opening near the rim of the chamber with one superfusate fraction collected every 5 minutes. Gills are first superfused with ASW for 30 minutes. Then, for the next 15 minutes ganglia are perfused with ASW containing 50 mM KCl alone (control) or with drug. Finally, the gills are again perfused with ASW for the remainder of the experiment. Radioactivity of the superfusate solutions is determined by liquid scintillation spectrometry. The values obtained represent the percentage of the total radioactivity in the ganglion that was released during the 5 minute period ending at the time indicated. Each value is the mean of five separate experiments. The standard error of the mean is less than 1.5 percent for all values. The ^{3}H release substance was analyzed with TLC to insure the material is either DA or 5-HT.

Determination of Biogenic Amines

For analysis of the content of biogenic amines, gills are combined and assayed for DA and 5-HT with or without neurotoxin treatments. The tissues are homogenized by a polytron (setting 4 for 10 s). Tests for DA are homogenized by a polytron radioenzymatic assay modified by Stefano and Catapane (1979). After homogenization and centrifugation a 10 µl aliquot of the supernatant is incubated for 1 h at 37°C with a medium containing: 50 µg of dithiolthreitol, 0.05 µM of $MgCl_2$; 14 µM of Tris-HCl buffer, pH 9.6, 1 µl of partially purified COMT, and 1 µl of [^{3}H]-methyl-S-adenosyl-L-methionine (SAM, 14.1 Ci/mM). After incubation the reaction vials are placed in an ice water bath and 0.5 µl of 0.5 M borate buffer, pH 10. The samples were then processed for TLC separation. Internal standards consisted of 40 µl aliquots of homogenate plus 10 µl of epinephrine, respectively. Blanks consisted of 40 µl of 0.2 N perchloric acid or tissue homogenate added to the incubation medium. The method described is able to detect 35 Pg of norepinephrine per 50 µl sample and 25 Pg of DA per 50 µl sample.

Histofluorescence

Tissues were frozen in isopentane cooled by liquid nitrogen and freeze dried for 4 days in an Edwards-Pierce System (EDT4). Tissues were then subjected to paraformaldehyde gas ($80^{\circ}C$, 1 hour) for primary amine fluorescence and then vacuum embedded with paraffin, followed by sectioning, activated by UV light and viewed for excitation at the green (catecholamine) and yellow (serotonin) wavelengths. Specific fluorescence is differentiated from nonspecific by: 1) Na^{+}-borohydride quenching, 2) fast fading (4-5 min), and finally, 3) treating tissues without paraformaldehyde. A Zeiss Epi-fluorescent Phase Contrast System coupled to a Zeiss Zonax Image Analysis system was employed. The sections were cut at 12 μm and the readings were consistently obtained at a gain of 10 and a h.v.537. The readings were corrected for nonspecific fluorescence as well as black current. Control values were set equal to 100 and treated tissues were compared to this level of intensity. Control values were obtained from 150 readings per slide and averaged with ten slides. The same applies for neurotoxin treated gills.

Ciliary Studies

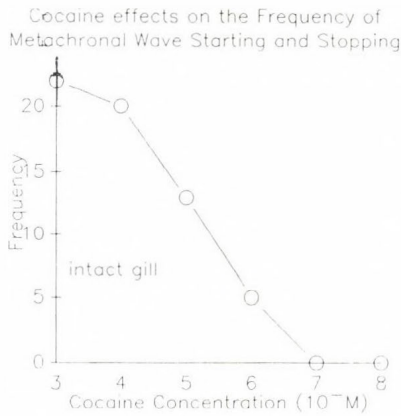
The animal was removed from the valves by cutting attachments of mantle and muscle and placed in artificial seawater (Aquarium Systems sea salts) at pH 7.8 and specific gravity 1024, similar to the average values for Long Island Sound water at this location. The mantle, foot and byssus were cut off and the animal was positioned on its dorsal surface with the gills extended to the sides. To improve microscopic observation of ciliary activity in the gill, the internal (medial) lamina was cut off and the animal tilted to that side to allow the gill to float relatively flat on the side to be observed. The animal was held in place by pinning through the posterior adductor muscle and between the anterior byssus retractors into a strip of rubber that had been glued to the bottom of the dish with Duco cement. Drug solutions were added to the medium bathing the gills which had their ganglionic connections severed

(branchial nerve). The preparation was washed liberally with artificial seawater to remove cellular debris and excess mucus and allowed to equilibrate for 1 hour before beginning the experiment.

The ciliary beating rate was determined by stroboscopic synchronization, using a General Radio Strobotac in place of the microscope lamp and observing the cilia with a 10x objective. Each value presented was obtained from 10 organisms (N) and measurements on 10 different filaments from each organism were made in quick succession and averaged to obtain one value for that preparation. Statistical comparison employed the one-tailed Student t test.

RESULTS

We have noted previously that the gill cilia have the ability to start and stop abruptly. Addition of cocaine to the gill bathing medium causes the gill cilia to increase the frequency of metachronal wave starting and stopping (Fig. 1). This phenomenon is dependent on the dose of cocaine employed. Reserpine addition to the gill medium causes the same effect (data not shown). Thus, the results suggest that cocaine caused the release of both DA and 5-HT from their respective nerves. In order to test this hypothesis we employed specific pharmacological lesioning agents. Incubating the gills in either 6-hydroxydopamine (6OHDA) or 5,6-hydroxytryptamine (5,6DHT) for one hour followed by a 6-day incubation in ASW appears to have removed the DA inhibitory activities and 5-HT excitatory activities in these gills (Fig. 2). Cocaine addition to the gills treated with 6OHDA caused only cilioexcitation, whereas cocaine addition to the 5-DHT treated gills would only cause cilioinhibition. These results indicate that cocaine may initiate the starting and stopping of the gill cilia by causing the release of the respective neurotransmitter. Reserpine application along with neurotoxin pretreatment caused the same effects as noted for cocaine.



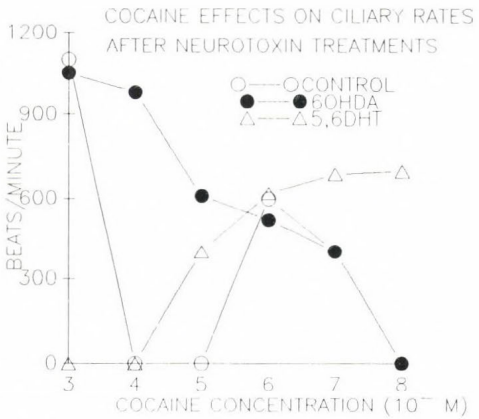
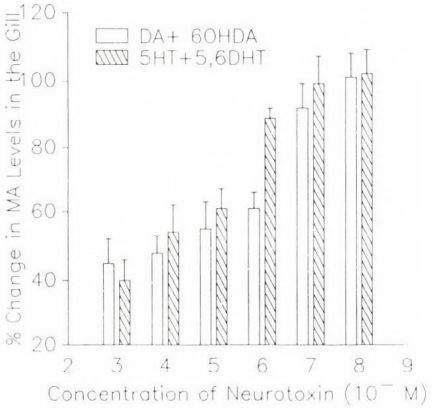


Fig. 1 (left). Effect of increasing concentrations of cocaine on the frequency of starting and stopping of the metachronal wave pattern. Each point represents 10 readings and the values did not vary by more than 5%.

Fig. 2 (right). Addition of cocaine to gills treated with neurotoxin (10^{-5} M 5,6DHT; 10^{-6} M 6OHDA) to demonstrate the effect after the chemical lesioning on the intact neurotransmitter system. Each point represents 12 readings and the values did not vary by more than 6%.

To confirm the physiological data, we estimated the levels of the respective monoamines both before and after neurotoxin treatment (Fig. 3). The gill content of both 5-HT and DA is 2.4 and 1.5 µg/g wet weight, respectively. Following the respective neurotoxin treatment (10^{-4} M) the values were reduced to 53 and 47% of the original monoamine levels, respectively. Interestingly, 5,6DHT was not as potent at the 10^{-6} M dose as was 6OHDA. The biochemical data were confirmed by analysing the fluorescent intensity emitted from the respective monoamines (Fig. 4). Utilizing this method we found that the specific fluorescence for both 5-HT and DA was reduced in the neurotoxin treated gills by 30 and 35%, respectively. In this analysis the fluorescent intensity was measured with a 20 µ diameter of the neural fibres in the vicinity of the chitin-like rod that runs in the gill filaments. Wavelength analysis confirmed both green and yellow spectra.

It is noteworthy to point out that both these methods indicate that the gill still contains measurable quantities of the respective monoamines. Yet, on a physiological level we were



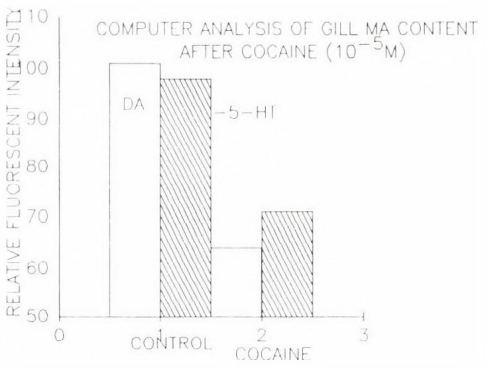


Fig. 3 (left). Biochemical levels of dopamine and serotonin following neurotoxin treatment. The serotonin content of the gill is 2.4 and dopamine is 1.5 µg/g wet weight. Values did not vary by more than 4%.

Fig. 4 (right). Computer analysis of the fluorescent intensity of the monoaminergic para-formaldehyde reaction product following cocaine pretreatment. Each value represents over 500 readings which did not vary by more than 7%.

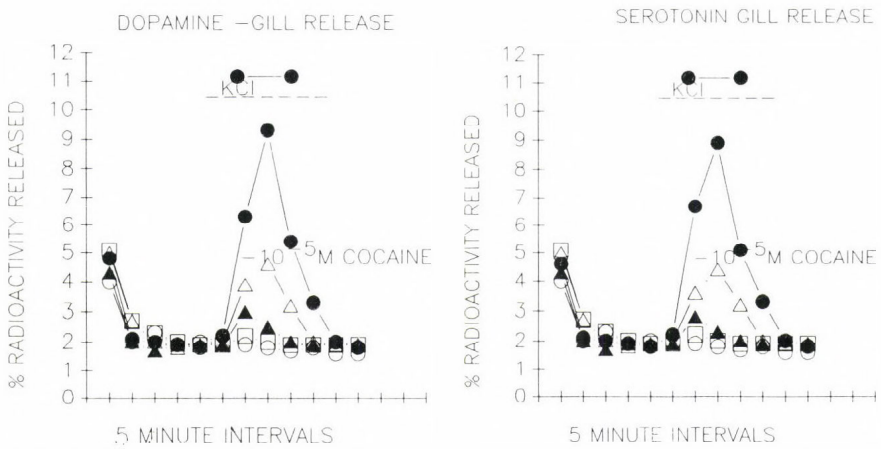


Fig. 5 (left), Fig. 6 (right). Effect of cocaine on the release of the biogenic amines. Closed circles - KCl induced release; Open circles - control; open triangles - cocaine; closed triangles - respective neurotoxin effect on the release induced by cocaine; open square - effect of the respective neurotoxin on the release of the biogenic amine. Each point represents the mean values obtained from five trials.

Table 1. Accumulation of dopamine and serotonin by the gill tissue of Mytilus edulis and their blockade by cocaine

	High affinity uptake system		
	Km_1 (μM)	V_{max1} (nmol/g/min)	ID_{50} (10^{-6} M)
Dopamine	2.25	1.144	
3-Chlorimiprimine			98.0
Benztropine			0.8
Cocaine			6.4
Serotonin	1.12	0.381	
3-Chlorimiprimine			1.2
Benztropine			137.0
Cocaine			8.1

The gill was incubated with the specified agents in addition to (^{3}H) amine for a period of 30 min. Results are expressed as ID_{50} (inhibition of 50% uptake) which were calculated from log probit plots of per cent change vs. concentration of substance used. Each point represents the mean value of 5 separate determinations for each concentration tested (best fit line by linear regression analysis).

able to obliterate the effects of the these monoamines with the use of the specific neurotoxin. This may indicate that the neural elements are being repaired, or that the toxin effects are highly localized in the terminal regions of the nerve fibres, thus specifically altering the release process in the respective neuron or a combination of the two. Examination of the release process which is believed to be affected by these treatments (Figs 5 and 6) demonstrated that cocaine can initiate the release of both monoamines. However the dose that results in release is rather high (10^{-5} M), in addition to causing only a slight release when compared to the effects of 50 mM KCl, approximately 4% to 9.2%, respectively. In gills treated with the respective neurotoxins, release could not be observed. Thus, the toxins appear to damage the site of neurotransmitter release.

The higher levels of 5-HT in the gill also may indicate that another source of 5-HT is present in the gill since the levels

of 5-HT are higher than those of DA. One source may be the amoeboid-type cells that are found in the haemolymph. These cells do exhibit specific 5-HT fluorescence (unpublished).

The literature concerning cocaine also makes note of the fact that it appears to block monoaminergic reuptake mechanisms. Since our data may also be viewed from the point of view of blocking the reuptake of either 5-HT or DA, we examined this mechanism. Table 1 demonstrates that cocaine is an effective, potent but nonspecific reuptake blocker for both DA and 5-HT. In addition, it appears to be a more potent inhibitor of reuptake than an inducer of neurotransmitter release. Yet, both activities may work together to increase the life of the transmitter molecule in the synaptic cleft.

DISCUSSION

The present study demonstrates that cocaine can exert profound effects on monoaminergic neural mechanisms in invertebrates. In addition, the effects are quite complex. The prime effect appears to be exerted on the high affinity reuptake mechanism of 5-HT and DA. By blocking the reuptake of both serotonin and dopamine these signal molecules are present in the synaptic cleft for longer periods of time, thus causing an increase in the frequency of starting and stopping of the gill cilia. This action of reuptake blockade is a principal mechanism used to explain the effects of cocaine in mammalian neural tissues (Hertting et al., 1961; Muscholl, 1961; Blackburn et al., 1967).

Interestingly, a secondary effect of cocaine also was demonstrated, namely, inducing the release of both neurotransmitters. Thus, both actions of this substance may be viewed as potentiating the effects of these signal molecules in the synapse, in this case the neuro-ciliary junction. In mammals cocaine also appears to enhance DA release (Farnebo and Hamberger, 1971; Costa et al., 1972).

In addition, the results of the present study serve to demonstrate that quite complex neural mechanisms can be found in

invertebrate neural tissues since the proposed mechanisms of action for cocaine suggested in this report appear to be similar to those described in the vertebrate literature. This in turn suggests that stability exists in regard to the evolution of the nervous system (Stefano, 1986).

ACKNOWLEDGEMENTS

The work was in part supported by Grants NIH-MBRS 08180 and ADAMHA-MARC 17138, The Long Island Community Foundation, Inc., Hoffmann-LaRoche, The Upjohn Company and the State University of New York Research Foundation. Mr. J. Sinisterra is an ADAMHA MARC Fellow, Ms. E. Braham is a NIH MBRS student, and Mr. L. Bentvena and Ms. J. DeFiglia are members of the undergraduate biomedical research program at SUNY at Old Westbury.

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DISCUSSION

ARVANOV, V.: Have you studied the effects of cocaine on Ca-uptake processes and on cyclic nucleotide system? Is it possible that cocaine-induced enhancement of ACh release is realized by one of these ways?

STEFANO, G.B.: No, at present it would be the released biogenic amines that would stimulate adenylate cyclase activity. The answer to your second question is no. I believe ACh does not directly stimulate ciliary beating since its application at 10^{-4} mol/l only stimulates this beating in 50% of the cases. Yet, in all cases it will stimulate gill filament movement which, I believe, leads in some cases to stimulatory ciliary activity as a side result.

ERDÉLYI, L.: Do you think that the effect of cocaine on dopamine and serotonin release is in correlation with any action of the drug on ionic channels or currents?

STEFANO, G.B.: No, as noted by the specificity at concentrations that are low enough to account for the local penetration barrier.

JANSE, C.: Cocaine causes a higher frequency of starting and stopping of the cilia beat. May this be due to fluctuations of 5HT and DA release?

STEFANO, G.B.: Yes, this is exactly what I have been saying. The release of both agents probably initiates this on-off-alternating phenomena.

LUKOWIAK, K.: Is the primary effect of cocaine on the functioning of the ciliary wave due to release of transmitter or to the blockade of its uptake?

STEFANO, G.B.: As noted by the effective concentration the 1^{o} effect appears to be blocking reuptake and 2^{o} inducing release. Thus, cocaine exerts a "one-two punch" by keeping the signal molecules in the synaptic cleft.

MOFFETT, S.: Do you think that the serotonin-reactive haematocytes may have accumulated serotonin by phagocytosis?

STEFANO, G.B.: No, I have noted them elsewhere in the organism. Their fluorescence is authentic and indeed they contain serotonin.

WINLOW, W.: Do serotonin- and dopamine-containing neurons make direct mono-synaptic connections to ciliated cells via neuro-ciliated cell junctions?

STEFANO, G.B.: I believe the junctions can be referred to as neurociliated ones. In addition, the data suggest that the contacts are in the centre of the gill filament where the metachronal waves appear to be starting.

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SOME ASPECTS OF MOLLUSCAN NEUROPHARMACOLOGY

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INTRODUCTION

The molluscan nervous system provides an excellent model for the analysis of small neuronal networks and receptor pharmacology. Central molluscan neurones, particularly those from gastropods, possess receptors for most if not all the known transmitters and their pharmacological properties have been reviewed recently (S.-Rózsa 1984; Walker 1986). In the present study we shall present current work on dopamine, adenosine and gamma-aminobutyric acid (GABA) receptors located on identified neurones from the land snail, Helix aspersa.

Dopamine has been shown to occur in gastropods and to be a central transmitter (Berry & Cottrell 1975). The inhibitory receptor has been best documented but dopamine also excites neurones and may have biphasic effects, usually excitation followed by inhibition. The inhibitory dopamine response is associated with an increase in conductance to potassium ions (Bokisch & Walker 1986) and the hyperpolarization when maximum approaches the equilibrium potential for potassium in these cells, that is, around -75 mV (Kostyuk 1968). There is clear evidence that dopamine acts on a specific dopamine receptor with properties distinct from an alpha or beta catecholamine receptor (Woodruff & Walker 1969).

Purines have been shown to modulate synaptic activity in the nervous system, for example, adenosine will depress evoked potentials and spontaneous activity in the mammalian brain (Dunwiddie & Hoffer 1980). Purines, for example ATP, have been shown to potentiate the action of acetylcholine potentials in amphibian sympathetic ganglion cells (Akasu & Koketsu 1985). They are believed to act at both pre- and postsynaptic sites and these different types of response have led to the classification of purine

receptors into P-1 and P-2 purinoceptor subtypes (Burnstock 1978). P-1 sites are mainly activated by adenosine and AMP while P-2 sites are activated by ATP and ADP.

GABA is an inhibitory transmitter throughout the animal kingdom. For example, it is a peripheral inhibitory transmitter in arthropods (Usherwood 1976) and a central inhibitory transmitter in vertebrates (Curtis & Johnston 1974). Gastropod central neurones are unusual as they possess GABA receptors which can be linked to sodium ionophores and so excite neurones (Yarowsky & Carpenter 1978; Bokisch & Walker 1986). Vertebrate GABA receptors have been classified into two types, GABA-A and GABA-B. The former are activated by muscimol and antagonised by bicuculline while the latter are activated by baclofen but are insensitive to both muscimol and bicuculline (Bowery et al 1981). The evidence for a transmitter role for GABA in molluscs is poor though there is evidence for glycine as a transmitter or modulator in Aplysia (Sawada et al. 1984).

MATERIALS AND METHODS

All experiments were performed on the land snail Helix aspersa which were collected locally and kept in the laboratory at room temperature. The brain was removed from the animal following the method of Walker (1968) and mounted on a glass slide and placed in a bath, volume 10ml. Recordings were made from cells identified from the map of Kerkut et al (1975). Glass microelectrodes were filled with molar potassium acetate or 3 molar potassium chloride, resistance in the range 10-30 MΩ. In many experiments square wave pulses were also applied so that changes in conductance could be monitored. Cell bioelectric potentials were amplified using conventional recording equipment including a Dagan 8100 single electrode clamp and displayed on either a Hewlett Packard or Brush pen recorder. Compounds were applied either directly to the bath or via a second pipette by ionophoresis. The saline used had the following composition: NaCl 100mM; KCl 4mM; $CaCl_2$ 7mM; $MgCl_2$ 5mM; Tris-HCl 5mM. Compounds added by bath addition were made up in Helix saline while those applied ionophoretically were made up in distilled water at a pH of 4.

RESULTS

Dopamine

Dopamine inhibits many Helix neurones including E-1/2 in the visceral ganglion and F-5/6, F-1 and cells in the F-30 area of the right parietal ganglion (Kerkut et al. 1975). This dopamine response has been analysed

using a range of agonists and antagonists. Ergometrine is the most potent antagonist since it will reduce the dopamine response when applied in the low nM range. Apomorphine and bromocriptine are less potent requiring high nM and low µM concentrations to block. The benzamide sulpiride will also reduce dopamine inhibition but again only in the low µM range. This compound shows stereospecificity to the dopamine receptor since the (-) isomer is about 10 times more active than the (+) isomer. Zetidoline, a substituted benzamide, and SCH 23390, a benzazepine, also antagonised dopamine responses in the low µM range. Two dopamine agonists, SKF 38393, a benzazepine, and LY 171555, an ergoline, have been tested on both F-5/6 and F-30 cells. On F-5/6 cells where dopamine is active in the low µM range, neither compound had much effect. However on F-30 cells where dopamine is active in the low nM range, SKF 38393 was inhibitory though LY 171555 was still devoid of dopamine-like activity, figure 1. In fact LY 171555 slightly excited the cell. In this example, noradrenaline excited the cell as did phenylephrine and octopamine. Since noradrenaline was about 100 times more potent than octopamine and phenylephrine mimicked the action of noradrenaline, this experiment presents evidence for an excitatory alpha catecholamine receptor, figure 1. However even SKF 38393 on F-30 is relatively inactive since the potency ratio compared to dopamine is greater than 10,000. Two further agonists with dopamine-like activity have been tested on F-30, 6,7-ADTN and 5,6-ADTN, both tetrahydronaphthalene derivatives. The 6,7 analogue has clear dopamine-like activity, being 25-100 times less potent than dopamine while the 5,6-analogue has no inhibitory action and may have slight excitatory effects.

The inhibitory action of dopamine has also been examined in terms of the possible involvement of both calcium and adenylate cyclase. For this part of the study cells F-5/6 were used. Addition of 1 µM dibutyryl cAMP produced a time dependent enhancement of the response to dopamine. The maximum response to dopamine was obtained 35 minutes following application of dibutyryl cAMP which then gradually declined to control values after about 2 hours. The diterpene, forskolin, 0.1 µM, also enhanced the response to dopamine with a time scale similar to dibutyryl cAMP. In contrast the adenylate cyclase inhibitor MDL 12,330A, 0.12 µM, depressed the response to dopamine after 10 minutes exposure and this slowly recovered following washing. In calcium free/2mM cobalt saline the dopamine response was enhanced while in the presence of low µM concentrations of 4-aminopyridine, the dopamine response was reduced reversibly. Tetraethylammonium at high

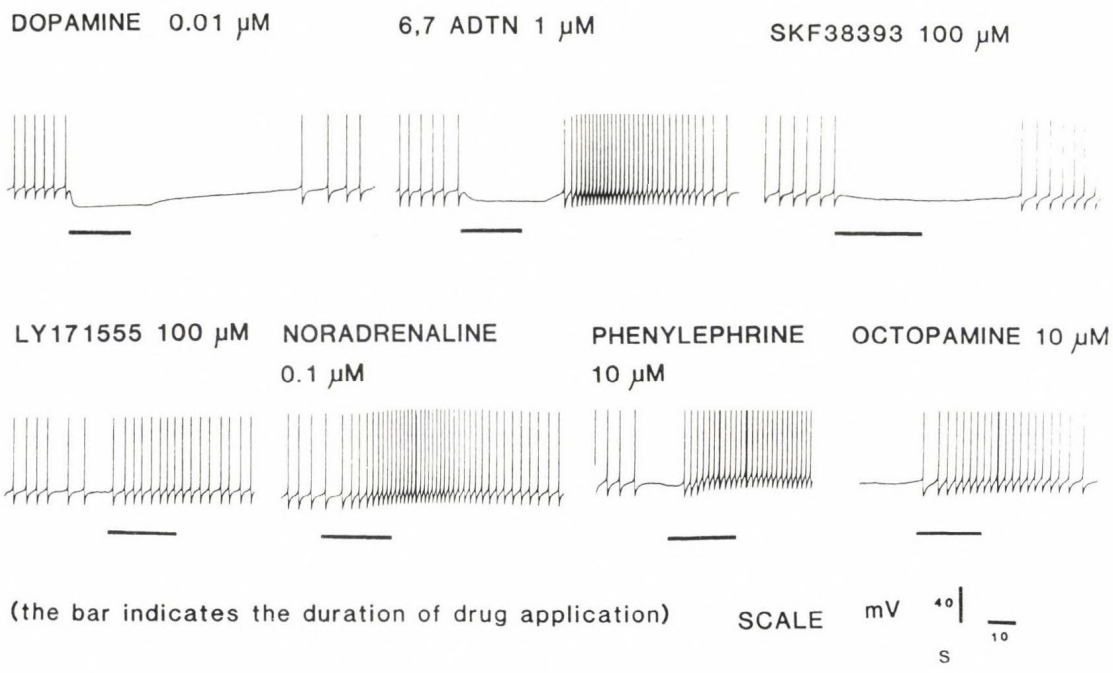

Figure 1. Recordings from a cell in the F-30 area of the right parietal ganglion of Helix. The cell is inhibited by dopamine, 6,7 ADTN and the D-1 agonist SKF 38393 but excited by noradrenaline, phenylephrine, octopamine and the D-2 agonist LY 171555.

μM concentrations irreversibly reduced the dopamine response while apamine, at low μM concentrations, actually enhanced the dopamine inhibition. When 7 mM barium was added to the saline instead of calcium, an action potential triggered prolonged periods of depolarization and when dopamine was applied it induced very large hyperpolarizations of up to 50 mV amplitude.

Adenosine

Acetylcholine depolarized and excited cell F-1 and the application of 60-600 nM adenosine reduced this depolarization. The effect of adenosine was readily reversed following washing. The relative potencies of L-PIA (N6-(L-2-Phenylisopropyl) adenosine) and NECA (5'-N-ethylcarboxamide-adenosine) were compared to adenosine in terms of this depression. L-PIA also depressed the acetylcholine response though it was slightly less potent than adenosine but NECA was inactive up to the highest concentration tested, 200 μM, Table 1. The effects of both adenosine and L-PIA were inhibited by 8-phenyltheophylline. In the presence of the adenosine uptake blocker, dipyridamole, 0.6 μM, the response to acetylcholine was reduced and in addition the depressing effect of adenosine on the acetylcholine response was enhanced, Table 1. Calcium is partly involved in the acetylcholine excitatory response and so the effect of raised calcium on the extent of the adenosine depression was investigated. In 20 mM calcium, the acetylcholine response was enhanced and the depressing effect of adenosine was also enhanced significantly in high calcium saline. Similarly in the presence of the calcium ionophore A23187 both the initial acetylcholine excitation was enhanced and the degree of depression by adenosine. When sodium-free/20 mM calcium saline was used the small residual response to acetylcholine was blocked by 3 mM cobalt or 50 μM verapamil, suggesting it was calcium mediated. This small response was almost completely blocked by adenosine. AMP but not ATP also depressed the acetylcholine excitation, Table 1.

Concentrations of adenosine in the low nM range, ie, 0.6 - 6 nM, enhanced the response to acetylcholine, Table 1. In addition to L-PIA, NECA in the range 0.6 nM - 0.2 nM, enhanced the acetylcholine excitation, Table 1. This effect of NECA reached a maximum at 10 nM and then plateaued. ATP and α, β-methylene ATP also enhanced the response to acetylcholine. Inosine and guanosine were inactive. Addition of 1 μM dibutyryl cAMP did not enhance acetylcholine until after washing. Enhancement of the acetylcholine response by NECA was not potentiated by dibutyryl cAMP.

Table 1. Summary of effects of purines on acetylcholine response of cell F-1. Relative enhancement and depression are expressed as pE and pA respectively. These values give a comparison of the ratios of the EC-50 values for acetylcholine in presence and absence of adenosine derivative divided by the concentration of the adenosine derivative.

Compound	Enhancement of Ach Response, pE (A-2) (Mean ± S.E.)	Depression of Ach Response, pA, (A-1) (Mean ± S.E.)
ADENOSINE	8.82 ± 0.18 (5)	6.94 ± 0.11 (5)
L-PIA	7.01 ± 0.14 (5)	5.79 ± 0.05 (5)
NECA	9.13 ± 0.01 (5)	-
(ADENOSINE + DIPYRIDAMOLE)	-	7.42 ± 0.19 (5)
AMP	-	6.57 ± 0.16
ATP	7.03 ± 0.11 (4)	-
α, β-METHYLENE ATP	6.22 ± 0.11 (5)	-
INOSINE	No effect 1nM - 10μM	
GUANOSINE	No effect 1nM - 10μM	

Gamma-aminobutyric acid (GABA)

The GABA cells used in these experiments were E15/17 in the visceral ganglion for excitation and cells E-4 and D-5 in the visceral and left parietal ganglia respectively for inhibition. The effects of a range of agonists were tested on both cell types and the results summarised in Table 2. As can be seen from the table there are distinct differences between the preferred requirements for activation of the GABA excitatory and inhibitory responses. For example, muscimol is 100 - 1000 times more potent than GABA at excitatory receptors but is a similar order of potency to GABA at inhibitory receptors. Similarly dihydromuscimol is far more active at the excitatory receptors than the inhibitory. Homomuscimol and thiomuscimol are of a similar potency to GABA at the excitatory receptors but inactive at the inhibitory receptors. Isomuscimol is inactive at both sites. The carboxylic and sulphonic acids of piperidine are both inactive at the inhibitory receptor but behave differently at the excitatory receptor. For example, piperidine-4-carboxylic acid is an agonist being around 13 times less active than GABA while the sulphonic acid is an antagonist. This antagonism appears to be selective since acetylcholine excitation is not altered. THIP is another restricted compound which clearly

Table 2. Equieffective Molar Ratio (EMR) values (mean ± S.E.) for some GABA analogues against both the excitatory and inhibitory responses to GABA; n = value in brackets.

Compound	'D' Response	'H' Response
GABA	1.0	1.0
Ethylenediamine	9.3 ± 2.5 (6)	92.3 ± 11.9 (6)
Muscimol	0.0013 ± 0.0004 (6)	0.4 ± 0.11 (5)
Dihydromuscimol	0.0072 ± 0.0008 (6)	0.5 ± 0.1 (4)
Homomuscimol	0.43 ± 0.05 (6)	> 100
Isomuscimol	> 100	> 100
Thiomuscimol	2.1 ± 0.16 (8)	> 100
Piperidine-4-carboxylic acid (P-4-C)	13.4 ± 1.4 (7)	> 100
Piperidine-4-sulphonic acid (P-4-S)	Antagonist	> 100
4,5,6,7-Tetrahydroiso-xazolo(5,4-c) pyridin-3-ol (THIP)	1.14 ± 0.24 (6)	> 100
Isoguvacine	1.8 ± 0.4 (9)	> 100
cis-4-Aminocrotonic acid	1.4 ± 0.46 (5)	> 100
Baclofen	inactive	inactive

distinguishes between the two receptor types, Table 2, as do isoguvacine and cis-4-aminocrotonic acid. Baclofen does not possess any clear GABA agonist activity at either site.

The properties of these two GABA responses have also been investigated using compounds which antagonise GABA at vertebrate sites. Bicuculline (a phtalide-isoquinoline alkaloid from Corydalis) methobromide, 10 - 1000 µM, failed to clearly antagonise the excitatory effect. However 10 µM bicuculline methobromide did reversibly block the inhibitory response to 50 µl of 100 µM GABA (effective bath concentration 5 µM). The inhibitory GABA responses returned to control values within 10 minutes of removal of bicuculline. In contrast picrotoxin antagonised both GABA inhibition and excitation. For example, 1 µM, picrotoxin completely blocked 5 µM GABA though the recovery times of the two responses differed. In the case of GABA inhibition, the response recovered to control values within 10 minutes washing. With the excitatory response, this had only partially recovered after 80 minutes washing. Pitrazepin, another GABA antagonist, did not antagonise either GABA excitation or inhibition. A fourth GABA antagonist, SR 95531 (a pyridazinyl-GABA derivative) 10 µM, reversibly blocked both

GABA excitation and inhibition. The responses had returned to control values within 10 minutes washing.

DISCUSSION

The present study presents further evidence regarding the properties of transmitter/modulator receptors and the ways in which they can be modified. For example, it would appear that at least some cholinoceptive neurones also possess adenosine receptors which can modify the intensity of the postsynaptic response. Adenosine receptors can be divided into two subtypes, A-1 and A-2 (Van Calker et al 1979). A-1 subtype is associated with depression of adenylate cyclase activity while the A-2 subtype is associated with potentiation of the activity. The adenosine analogue L-PIA is more potent than NECA at A-1 sites while the reverse is true at A-2 sites (Daly 1982). Since NECA is inactive at depressing acetylcholine responses on cell F-1 of Helix it would appear that this response is of the A-1 type. Conversely since NECA is more potent than L-PIA at potentiating the same acetylcholine response it would appear that this potentiation is linked to an A-2 subtype. Although application of dibutyryl cAMP did enhance the acetylcholine response following washing it failed to increase the potentiation due to NECA. Further studies are required to determine whether a cyclase is involved in these events. Since 8-phenyltheophylline antagonised the depressant effects of both adenosine and L-PIA, this receptor could be termed a P-1 type. The present experiments also suggest that adenosine is interacting with calcium and hence modifying the acetylcholine response. There is evidence that adenosine depresses activity by antagonising calcium dependent potentials in dorsal root ganglion neurones (MacDonald et al 1986). Interestingly in this preparation they found that NECA was inactive. The link between adenosine depression of the F-1 acetylcholine excitatory response and calcium suggests that the receptor mediating this depression might belong to the A-3 subtype proposed by Ribeiro and Sebastiao (1986). Adenosine potentiation of the acetylcholine response is probably associated with an action at an allosteric site of the acetylcholine receptor-sodium channel complex. For example, ATP can activate a sodium conductance in neurones from rat and cat sensory ganglia (Krishtal et al 1983).

Dopamine responses can be classified into D-1 and D-2 subtypes (Kebabian & Calne 1979). The D-1 type is linked to adenylate cyclase. The benzazepines are the best pharmacological probes for this subtype. For example,

SKF 38393 is a selective D-1 agonist (Setler et al.1978) while SCH-23390 is a selective antagonist (Hyttel 1983). Since SKF 38393 will mimic the inhibitory action of dopamine on Helix neurones and SCH-23390 will antagonise it, the receptor would appear to be of the D-1 type. In addition the response is clearly linked to a cyclase since dibutyryl cAMP will greatly potentiate it. Further evidence for this comes from the observation that the D-2 receptor agonist LY 171555 is devoid of activity. However, the D-2 antagonist sulpiride does block dopamine at this site. Other evidence in favour of dopamine inhibition on Helix neurones being a D-1 type is that apomorphine is an antagonist, as are the ergot alkaloids. In contrast to these findings Stoof et al.(1985) found evidence for dopamine inhibition on the Growth Hormone neurones of Lymnaea to be mediated by a D-2 receptor. Dopamine excitation on these cells appeared to be via a D-1 receptor. Clearly gastropod dopamine receptors are not homogeneous. In terms of the preferred conformation for dopamine receptor activation the Helix inhibitory receptor appears to be similar to the mammalian receptor since it is activated by 6,7-ADTN while 5,6-ADTN is inactive.

The finding that on certain F-30 neurones, noradrenaline, phenylephrine and octopamine are excitatory but that noradrenaline is more potent than octopamine would suggest the presence of alpha catecholamine receptors. There are very few examples of alpha receptors in invertebrates and if subsequent experiments verify this observation it will be of considerable interest.

The ionic mechanism associated with dopamine inhibition on Helix neurones is linked to potassium. The present work suggests that this potassium event is not linked to calcium.

GABA receptors can be classified into GABA-A and GABA-B subtypes based on their responses to muscimol, bicuculline and baclofen (Bowery et al. 1981). Since both the excitatory and inhibitory GABA receptors on Helix neurones are activated by muscimol and not by baclofen they would appear to be of the GABA-A subtype. Bicuculline reversibly blocked the inhibitory but not the excitatory response which provides further evidence that the inhibitory response resembles the vertebrate GABA-A receptor. However the structure activity studies described show there are clear differences both between the two Helix receptors and between them and the vertebrate GABA-A receptor. For example, the GABA-A receptor in vertebrates is activated by THIP and isoguvacine, both compounds are inactive on Helix inhibitory GABA responses. Piperidine-4-sulphonic acid is a vertebrate GABA-A agonist but

is an antagonist on Helix GABA excitatory receptors and is inactive on Helix GABA inhibitory receptors. Another interesting finding is that picrotoxin, which blocks GABA inhibition at the level of the chloride ionophore (Constanti 1978), blocks both Helix GABA responses. While the Helix GABA inhibitory response is chloride mediated, the excitatory response is largely associated with sodium (Bokisch & Walker 1986). Does picrotoxin block the sodium ionophore linked to GABA excitation or does it block the GABA excitatory receptor directly? A difference between the action of picrotoxin on Helix GABA excitatory and inhibitory responses is the time for recovery. In the case of the chloride mediated inhibition, picrotoxin block is quickly reversed while picrotoxin at the same concentration only very slowly reversed at Helix GABA excitatory receptors. Both Helix GABA receptors respond in the same way to two other GABA antagonists, pitrazepin which is devoid of blocking action and SR 95531 which blocks both reversibly with the same time course of recovery. Yarowsky & Carpenter (1978) in their study on GABA receptors of Aplysia neurones, identified 6 different responses, 4 producing depolarizations and 2 hyperpolarizations. They found picrotoxin and bicuculline blocked only the chloride mediated inhibition. Baclofen has been found to inhibit neurones in Onchidium through an increase in potassium conductance (Shimizu et al. 1983). It would be interesting to investigate the pharmacology of this baclofen sensitive inhibitory response in Onchidium.

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DISCUSSION

FLOREY, E.: What is your evidence that the excitatory responses to GABA are due to a "sodium-mechanism"? If the effect is Na-dependent, is it not possible that it is due to a decrease of K-conductance?

WALKER, R.J.: The GABA excitation we studied on cells E15/17 is associated with an increase in conductance and disappears in Na-free saline. In fact, a small inhibition is often revealed in Na-free saline suggesting that GABA excitation is associated with an increase in permeability to both Na and Cl ions.

KLEE, M.R.: Neurons of the Japanese sea-snail Onchidium possess $GABA_B$ and $GABA_A$ receptors; the $GABA_B$ response being Ca^{++}-dependent (Shimizu et al., Jap. J. Physiol., 1983).

WALKER, R.J.: Yes, I know of this paper but if I remember correctly the baclofen response was rather unusual and did not exactly mimic the GABA action.

LUKOWIAK, K.: 1. Janet Richmond in my laboratory has now demonstrated a $GABA_B$ type receptor. It is baclofen sensitive.

2. What role might the adenosine receptors play in the behaviour of the animal. Do they have any physiological role?

WALKER, R.J.: 1. I am very pleased that you have found a $GABA_B$ receptor on Heliosoma neurones. This further confirms the value of gastropods as model systems for receptor analysis. I was sure that sooner or later a $GABA_B$ receptor would be identified on a gastropod neurone.

2. I assume that the adenosine receptors are postsynaptic and while being separate from the acetylcholine receptors they are closely associated with them. The acetylcholine excitatory response is linked to Na and Ca channels and the extent of the activation of these channels by acetylcholine can be modified by adenosine. Low concentrations of adenosine enhance the acetylcholine response by increasing the efficacy of acetyl-

choline to activate Na channels while higher concentrations of adenosine depress the response at the level of the Ca channels. I do not know how this is achieved but our experiments show that adenosine depresses the Ca component of the acetylcholine excitatory response.

STEFANO, G.B.: In reference to using antagonists in 100 µM concentrations have you tried them with other transmitter systems to indeed indicate the specificity?

WALKER, R.J.: At the concentration of 100 µM, I am sure that sulpiride would blook 5-HT. In our study, we had to use high concentrations to obtain clear antagonism to dopamine. We obtained the same antagonism with the D-1 antagonist SCH 23390, confirming they are not selective at these concentrations on invertebrate dopamine receptors.

TAUC, L.: What precautions do you take to rule out that the responses are caused by transsynaptic effect of the drugs?

WALKER, R.J.: This is difficult to answer but from experience of working with identified cells and a combination of applying compounds by ionophoresis and bath application, it is possible to normally distinguish between direct and indirect actions via interneurones. But it is a serious problem and one must always be aware of it.

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THE PHARMACOLOGICAL CHARACTERISTICS OF β-HYDROXY-L-GLUTAMIC ACID (L-BHGA) RECEPTORS IN IDENTIFIABLE GIANT NEURONES OF ACHATINA FULICA FERUSSAC

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INTRODUCTION

We have identified about 30 giant neurones in the ganglia of an African giant snail (Achatina fulica Férussac). Although L-glutamic acid (L-Glu) is thought to be an important excitatory neurotransmitter in the mammalian central nervous system, none of the identifiable giant neurones of Achatina fulica Férussac was sensitive to L-Glu. However, several Achatina neurones were found to be sensitive to β-hydroxy-L-glutamic acid (L-BHGA) (Takeuchi et al., in press).

In this report, we summarize the results obtained on the pharmacological characteristics of L-BHGA receptors in these Achatina giant neurones by testing the stereoisomers of BHGA, the glutamate analogues having cyclopentane ring, those having cyclopropane ring, the sulfur-containing amino acids, etc. (Watanabe et al., 1985; Nakajima et al., 1985; Novales-Li et al., in press).

MATERIALS AND METHODS

African giant snails (Achatina fulica Férussac) were collected in Davao and Manila (Philippines), and brought by air to Japan. The following five giant neurones, sensitive to L-BHGA, were used in this report: PON (periodically oscillating giant neurone), VIN (visceral intermittently firing neurone), d-RPLN (dorsal-right parietal large neurone), RAPN (right anterior pallial neurone) identified in the

suboesophageal ganglia; and v-RCDN (ventral-right cerebral distinct neurone) identified in the cerebral ganglia (Takeuchi et al., 1976; Takeuchi and Yamamoto, 1982; Ku and Takeuchi, 1983a; b; 1985). The localization of these neurones is shown in Fig. 1.

We examined the effects of the following compounds: (S)-(+)-glutamic acid (L-Glu) and (R)-(-)-glutamic acid (D-Glu) (donated by Central Research Laboratories of Ajinomoto Co., Inc., Japan); (2S,3S)-(+)-ß-hydroxyglutamic acid (erythro-L-BHGA), (2S,3R)-(+)-ß-hydroxyglutamic acid (threo-L-BHGA), (2R,3S)-(-)-ß-hydroxyglutamic acid (erythro-D-BHGA), (2R,3R)-(-)-ß-hydroxyglutamic acid (threo-D-BHGA), (2S,3S,4S)-2-carboxy-4-isopropenylpyrrolidine-3-acetic acid (α-kainic acid), (2S,3S,4R)-2-carboxy-4-isopropenylpyrrolidine-3-acetic acid (α-allo-kainic acid), (2S,3S,4S)-2-carboxy-4-(1-methyl-5(R)-carboxy-1-(Z),3(E)-hexadienyl)pyrrolidine-3-acetic acid (domoic acid), erythro-L-α-amino-3-oxo-isoxazolidine-5-acetic acid (erythro-L-tricholomic acid), DL-α-amino-3-oxo-4-isoxazoline-5-acetic acid (DL-ibotenic acid), L-α-amino-ß-(3,5-dioxo-1,2,4-oxadiazolidine-2-yl)-propionic acid (L-quisqualic acid), (2S,3S,4S)-(+)-α-(carboxycyclopropyl) glycine (erythro-L-ACCG-extended), (2S,3R,4R)-(-)-α-(carboxycyclopropyl)glycine (threo-L-ACCG-extended), (2S,3S,4R)-(+)-α-(carboxycyclopropyl)glycine (erythro-L-ACCG-folded), (2S,3R,4S)-(+)-α-(carboxycyclopropyl)glycine (threo-L-ACCG-folded), (2S*,3S*,4S*)-N-t-butoxycarbonyl-α-(carboxycyclopropyl)glycine (erythro-DL-N-Boc-ACCG-extended), (2S*,3S*)-α-(methylenecyclopropyl)glycine (erythro-DL-AMCG), (2S*,3R*)-α-(methylenecyclopropyl)glycine (threo-DL-AMCG) and (-)-cyclopropylalanine ((-)-CA) (synthesized or isolated in our laboratories); and L-homocysteic acid (L-HCA) and L-homocysteine sulfinic acid (L-HCSA) (donated by Professor S. Ohmori of Okayama University, Japan).

These compounds were dissolved in the snail's physiological solution (Takeuchi et al., 1973), and administered by way of bath application. The screening trials were conducted at the concentration of 10^{-3} M. The chemical structures of these compounds are presented in Figs. 2-4.

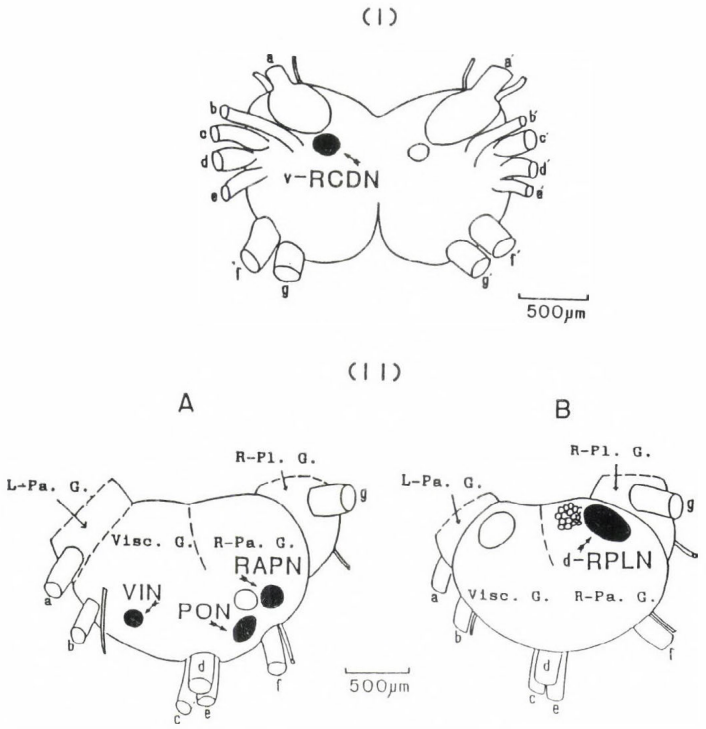

Fig. 1. Localization of identifiable giant neurones sensitive to ß-hydroxy-L-glutamic acid (L-BHGA) in ganglia of Achatina fulica Férussac. I. Cerebral ganglia. In I, v-RCDN, ventral-right cerebral distinct neurone. II. Sub-oesophageal ganglia. In II, L-Pa. G., left-parietal ganglion; Visc. G., visceral ganglion; R-Pa. G., right parietal ganglion; R-Pl. G., right-pleural ganglion. A, Caudal part. In A, PON, periodically oscillating neurone; RAPN, right anterior pallial neurone; VIN, visceral intermittently firing neurone. B, Rostral part. In B, d-RPLN, dorsal-right parietal large neurone. Scale, 500 um.

To compare the potency of each compound with those of others, its effective potency quotient (EPQ) vis-à-vis the potency of the more effective L-BHGA isomer was calculated, by the proportion of the minimum effective concentration (MEC) of the more effective L-BHGA isomer to that of the given compound.

RESULTS

Effects of L-BHGA stereoisomers

As shown by their chemical structures in Fig. 2, ß-hydroxyglutamic acid (BHGA) has four (erythro-L-, threo-L, erythro-D- and threo-D-) stereoisomers. Table 1 summarizes the effects of these BHGA isomers on the five giant neurones, together with those of L-Glu and D-Glu (Watanabe et al., 1985).

L-glutamic acid (L-Glu) and D-Glu had no marked effects on the five giant neurones tested; the two Glu isomers showed slight effects only on RAPN and v-RCDN (EPQs vis-à-vis potency of more effective L-BHGA: 0.1 for RAPN; 0.01-0.03 for v-RCDN). Of the four BHGA stereoisomers, the two L-BHGA isomers were in general more potent than those of D-BHGA. Of the two D-BHGA, threo-D-BHGA was rather more potent on some neurones, for example, d-RPLN and RAPN (EPQ: 0.3). On the other hand, the two L-BHGA isomers had marked effects on all of the neurones tested.

Of the two L-BHGA isomers, erythro-L-BHGA was more effective on PON, VIN and d-RPLN than threo-L-BHGA. On the other hand, threo-L-BHGA was more potent on v-RCDN. The two L-BHGA isomers were equally effective on RAPN. Based on these

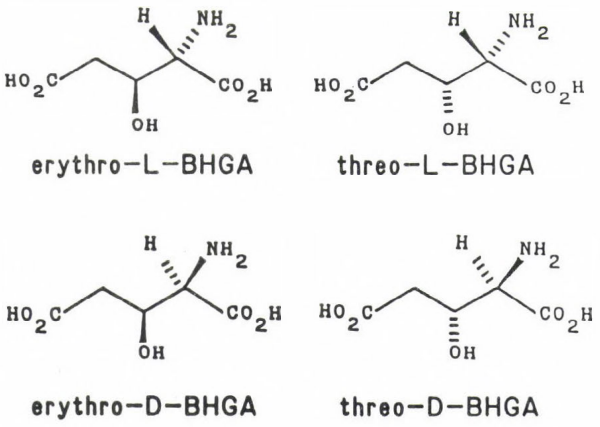

Fig. 2. Chemical structures of four L-BHGA stereoisomers.

findings, we categorized the neurones as follows: PON, VIN and d-RPLN in the erythro type; RAPN in the combined type; and v-RCDN in the threo type. The minimum effective concentrations (MECs) of the more effective L-BHGA were 10^{-5} - 10^{-4} M.

Effects of glutamate analogues having cyclopentane ring

Glutamate analogues having cyclopentane ring, chemical structuresof which are shown in Fig. 3, were originally isolated from natural products; α-kainic acid and domoic acid were isolated from seaweeds, Digenea simplex and Chondria armata, respectively; erythro-L-tricholomic acid and DL-ibotenic acid from mushrooms, Tricholoma muscarium and Amanita strobiliformis; and L-quisqualic acid from seeds of a plant, Quisqualis indica (Takemoto, 1978). Table 2 summarizes the effects of these compounds on the five giant neurones (Nakajima et al., 1985).

α-Kainic acid and domoic acid were effective on d-RPLN (EPQ: 0.3) and RAPN (EPQ: 1), which are excited by L-BHGA. DL-ibotenic acid was effective especially on PON and VIN (EPQ for

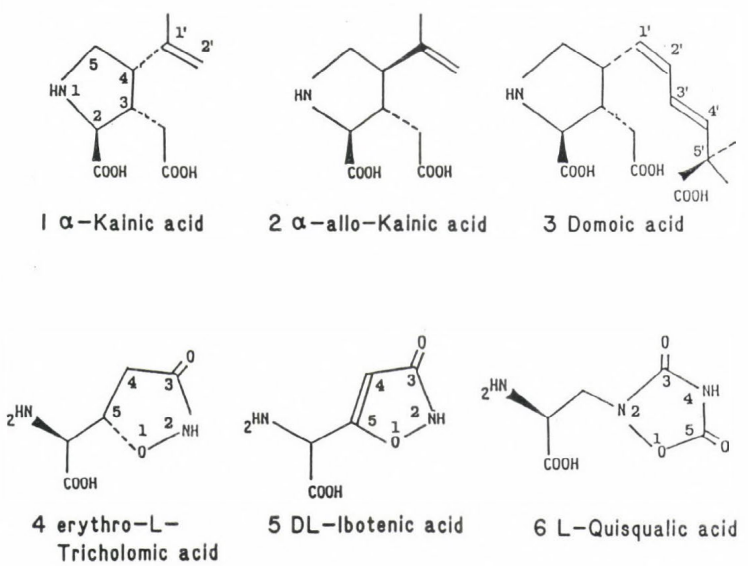

Fig. 3. Chemical structures of glutamate analogues. I. Compounds having cyclopentane ring.

TABLE 1

Effective potency quotient (EPQ) of stereoisomers of ß-hydroxyglutamic acid (BHGA) and glutamic acid (Glu) on five neurones of Achatina fulica Férussac (screening test at 10^{-3} M).

Type	---------- erythro ---------			combined	threo
Neurone	PON	VIN	d-RPLN	RAPN	v-RCDN
Effects of BHGA	I	I	E	E	I
No. Substance					
1. Erythro-L-BHGA	1	1	1	1	0.1
2. Threo-L-BHGA	0.3-0.1	0.3-0.1	0.1	1	1
3. Erythro-D-BHGA	< 0.03	0.1-0.03	(-)	(-)	0.03
4. Threo-D-BHGA	< 0.03	0.3-0.1	0.3	0.3	0.01
5. L-Glu	(-)	(-)	(-)	0.1	0.01
6. D-Glu	(-)	< 0.03	(-)	0.1	0.03
7. MEC of the most effective compound	$3x10^{-5}$	$3x10^{-5}$	10^{-4}	10^{-4}	10^{-5}

E, excitatory effects. I, inhibitory effects. (-), no effects at screening concentration (EPQ: 0). MEC, minimum effective concentration.

TABLE 2

Effective potency quotient (EPQ) of glutamate analogues having cyclopentane ring on giant neurones of Achatina fulica Férussac (screening test at 10^{-3} M).

Type	---------- erythro ---------			combined	threo
Neurone	PON	VIN	d-RPLN	RAPN	v-RCDN
Effects of L-BHGA	I	I	E	E	I
No. Substance					
1. α-Kainic acid	(-)*	(-)*	0.3	1	(-)*
2. α-Allo-kainic acid	0	<0.03	0	0	0
3. Domoic acid	(-)*	0.1-0.03	0.3	3-1	(-)*
4. e-L-Tricholomic acid	0.1	0.3	0.1	3-1	(-)*
5. DL-ibotenic acid	1	1	<0.1	1-0.3	(-)*
6. L-Quisqualic acid	3-1	1-0.3	1	30-10	3

E, excitatory effects. I, inhibitory effects. (-), no effects at screening concentration. (-)*, slight effects opposite to those of L-BHGA.

both neurones: 1), which are inhibited by L-BHGA. On the other hand, L-quisqualic acid was more effective on all of the neurones tested than L-BHGA; this compound was the most effective on RAPN (EPQ: 30-10).

Besides, Novales-Li (in press) demonstrated that threo-ß-hydroxy-DL-aspartic acid (threo-DL-BHAA) was effective on d-RPLN (EPQ: 1) and RAPN (EPQ: 0.3)

Effects of glutamate analogues having cyclopropane ring

α-Carboxycyclopropylglycine (ACCG) compounds are glutamate analogues, the conformations of which are fixed in either extended or folded forms by a cyclopropane ring (Fig. 4). The effects of the two sulfur-containing amino acids, L-HCA and L-HCSA, were also examined; the conformations of the two amino acids are considered to be in folded form. Effects of the stereoisomers (erythro- and threo-) of L-ACCG fixed in either extended or folded forms on the five giant neurones are summarized in Table 3.

According to the sensitivities to erythro-L-ACCG-extended and threo-L-ACCG-folded, we can classify these L-BHGA receptors into ß-hydroxy glutamate receptor class BHG 1, BHG 2 and BHG 3.

PON and VIN are classified under BHG 1; they were sensitive to both erythro-L-ACCG-extended (EPQ: 100-30) and threo-L-ACCG-folded (EPQ: 0.3-3); but almost not sensitive to threo-L-ACCG-extended and erythro-L-ACCG-folded. The two neurones were also sensitive to L-HCA and L-HCSA. On the other hand, d-RPLN and RAPN are classified under BHG 2; these are affected only by threo-L-ACCG-folded (EPQ: 0.3), and not so sensitive to other L-ACCG stereoisomers (EPQs: <0.1). The two neurones were also sensitive to L-HCA and L-HCSA.

Finally, v-RCDN, classified under BHG 3, was not so sensitive to all the stereoisomers of L-ACCG and the two sulfur-containing amino acids.

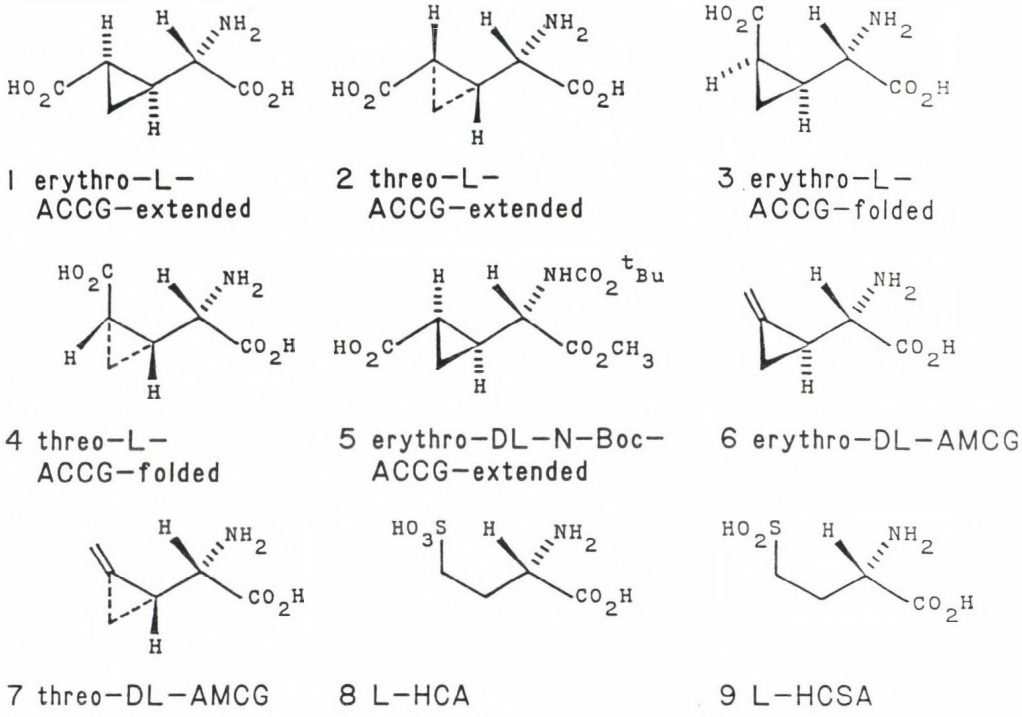

Fig. 4. Chemical structures of glutamate analogues. II. Compounds having cyclopropane ring.

TABLE 3

Effective potency quotient (EPQ) of glutamate analogues having cyclopropane ring on giant neurones of Achatina fulica Férussac (screening test at 10^{-3} M).

Classification	--- BHG 1 ---		---- BHG 2 ----		- BHG 3 -
Neurone	PON	VIN	ß-RPLN	RAPN	v-RCDN
Effects of L-BHGA	I	I	E	E	I
Erythro or threo types	erythro	erythro	erythro	combined	threo
No. Compounds					
1. erythro-L-ACCG-extended	30	100-30	0.1	(-)*	0.1
2. threo-L-ACCG-extended	0.03	0.03	(-)	(-)	(-)
3. erythro-L-ACCG-folded	(-)	(-)	0.1	<0.1	0.01
4. threo-L-ACCG-folded	3-1	0.3	0.3	0.3	(-)*
5. erythro-DL-N-Boc-ACCG-extended	(-)	(-)	(-)	(-)	(-)
6. erythro-DL-AMCG	(-)	(-)	0.3-0.1	0.1	(-)
7. threo-DL-AMCG	(-)	(-)	(-)	<0.1	(-)
8. L-HCA	3	3	1	0.3	0.01
9. L-HCSA	1	1	0.3	1	0.03-0.01

E, excitatory effects. I, inhibitory effects. (-), no effect at screening concentration. (-)*, slight effects opposite to those of L-BHGA. BHG 1-3, ß-hydroxyglutamate receptor class 1-3.

DISCUSSION

The functional roles of ß-hydroxyl group in L-BHGA and its agonists either in producing or preventing the effects of these compounds are considered as follows: in producing the effects (1) by fixing the conformation, or (2) as a proton donor or acceptor; and in preventing the effects (3) by occupying a space against the receptor. Of the L-BHGA analogues examined, L-ACCG compounds are able to be effective (1) by fixing their conformations; and able to prevent the effects (3) by occupying a space against the receptors; but these compounds cannot affect the receptors (2) as proton donor or acceptor.

According to the sensitivity of the BHG 1 receptors to L-ACCG compounds, the receptors may be composed of the two components, BHG 1A and BHG 1B. The BHG 1A receptors accept L-BHGA and erythro-L-ACCG-extended, and reject threo-L-ACCG-extended, erythro-L-ACCG-folded and possibly threo-L-ACCG-folded; these receptors are concluded to be sensitive to erythro-L-compounds in extended form. Since the effects of erythro-L-ACCG-extended on the BHG 1A are more potent than L-BHGA, the role of ß-hydroxyl group to produce the effects on the receptors may be "fixing their conformations". On the other hand, the BHG 1B receptors accept threo-L-BHGA, threo-L-ACCG-folded, but reject threo-L-ACCG-extended, erythro-L-ACCG-folded and maybe erythro-L-ACCG-extended; these receptors will be sensitive to threo-L-compounds in folded conformation. In addition, both L-HCA and L-HCSA may act on BHG 1B, since the two amino acids may be in folded form. Since the effects of threo-L-ACCG-folded are more potent on the BHG 1B receptors, the role of the hydroxyl group on the receptors may be also "to fix the conformation". The BHG 1 receptors are also sensitive especially to DL-ibotenic acid.

Less evidence was obtained with respect to the BHG 2 receptors than those of BHG 1 mentioned above. However, the BHG 2 receptors may be also composed of two kinds of receptors: BHG 2A and BHG 2B. The BHG 2A receptors accept only erythro-L-BHGA, but reject all isomers of L-ACCG. On the other

hand, the BHG 2B receptors accept threo-L-BHGA and threo-L-ACCG-folded; the receptors accept threo-L-compounds in folded form. Since threo-L-ACCG-folded is equipotent on these receptors, the role of the hydroxyl group may be "to fix the conformation". L-HCA and L-HCSA also act on BHG 2B receptors. The BHG 2 receptors are also sensitive to α-kainic acid, domoic acid and threo-DL-BHAA.

The BHG 3 receptors accept only threo-L-BHGA, but reject all of L-ACCG isomers. Their features are still obscure.

SUMMARY

None of the identifiable giant neurones of Achatina fulica Férussac are sensitive to L-glutamic acid (L-Glu), but the following neurones of the same snail are affected by β-hydroxy-L-glutamic acid (L-BHGA): PON, VIN, d-RPLN, RAPN and v-RCDN. The L-BHGA receptors are classified by their sensitivities to L-BHGA analogues. The BHG 1 receptors, which appear in PON and VIN and are linked with inhibitory ionic channels, are composed of BHG 1A and BHG 1B. The BHG 1A accept erythro-L-compounds in extended form, and the BHG 1B accept threo-L-compounds in folded form. The BHG 2 receptors, which appear in d-RPLN and RAPN and are linked with excitatory ionic channels, may also be composed of BHG 2A and BHG 2B. The BHG 2A accept erythro-L-compounds, and the BHG 2B accept threo-L-compounds in folded form. The BHG 3 receptors, which appear in v-RCDN and are linked with inhibitory ionic channels, accept threo-L-compounds; the features of BHG 3 are still obscure.

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ANALYSIS OF VERATRINE EFFECTS ON HELIX NEURONS: A POSSIBLE EPILEPSY MODEL

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INTRODUCTION

It has been known for more than 100 years that extracts from Veratrum species have strong physiological effects. This extract, veratrine, contains a group of steroid alkaloids, veratridine and cevadine being the major constituents (6). The experimental study of veratrine effects on excitable tissues commenced in the twenties of this century. Riesser (12) found that veratrine at low concentrations altered the muscle contractions in preparations from a wide variety of invertebrate species. They became longer and/or stronger and in certain cases repetitive twitches occurred. As judged from the nature of electro-mechanical coupling in smooth muscles, these phenomena indicate altered electrical properties of the muscle membrane. Similar effects of veratrine on vertebrate skeletal muscle and heart have been described (7). Contractures lasting several minutes or tens of minutes were observed. More recent findings concerning the electrical behaviour of the muscle membrane have been published by the Varga group (3), using mainly cevadine. They found a massive depolarization lasting for tens of minutes accompanied by a strong sodium influx (4).

The action of Veratrum alkaloids on neural elements has also been investigated by several authors. Squid axons under the effect of 20 /ug/ml veratridine were found to produce lasting depolarizations triggered by a spike (8). Alternatively, spike potentials were followed by strong and slowly decaying after-depolarizations, which, in certain cases, gave rise to repetitive discharge. Ranvier nodes of frog sciatic nerve responded to veratridine in a similar manner. Ulbricht (15) described steady membrane depolarizations and depolarizing afterpotentials following spike discharges. The current-

voltage characteristic changed to an N-shaped type. Both this author and Straub (13) noted that all the effects of veratridine were bound to the presence of sodium.

The influence of veratridine on snail neurons has been studied by Leicht et al. (8). According to their findings, 10 /ug/ml veratridine evoked long-lasting depolarization and sometimes oscillations of the membrane potential. Action potentials were followed by long after-depolarizations. The current-voltage curve displayed a region of negative slope resistance. These phenomena turned out to be sodium-dependent but were contrary to findings on muscle membranes, insensitive to tetrodotoxin. Slowly developing, long-lasting inward currents were described under effect of veratridine in voltage clamp conditions (9).

Considering the results reviewed above, we thought it promising to examine the effects of veratrine on Helix neurons from the point of view of experimental epilepsy. On the basis of their hyperactivity- and burst-inducing properties, these alkaloids can be considered as convulsants and may be suitable for creating models of epileptic phenomena at cellular level.

METHODS

Preparation

The experiments were done on the suboesophageal ganglion group of Helix pomatia. The ganglion was dissected out of the animal and the connective tissue was removed under Ringer's solution. The preparation was fixed with steel needles to the Sylgard bottom of a translucent chamber, being continuously perfused at a rate of 1 ml/min. It was illuminated from below and viewed through a stereomicroscope. The electrode was positioned by means of a mechanical manipulator.

Registration

A single microelectrode with resistance of 2-6 MOhm, filled with KCl-K-citrate 1:1 M, was introduced into the cell. The electric activity was led to a single-electrode device suitable for current- and voltage clamp operation. Command pulses were given by a square- and ramp-pulse generator. Output signals were fed into a Tektronix storage oscilloscope and an X-Y

plotter (for drawing current-voltage curves). The sampling cycle of the voltage clamp was continuously monitored on a separate scope. For optimal clamping and time resolution, sampling frequency, time constant and gain had to be set for each neuron. Drugs were applied to the neurons by continuous perfusion. Experiments were done at room temperature.

The neuronal activity was visualized, stored and photographed on the Tektronix screen. Current-voltage curves were drawn by the X-Y plotter in voltage clamp mode, using slow (20-25 mV/s) ramp pulses. Part of the records was corrected for leakage. Currents obtained with equal de- and hyperpolarizations from holding potential were evaluated and summed algebraically. The leakage was supposed to be linear. At I-V curves, the correction was done at every 10 mV on the active, depolarizing limb and the curve was redrawn according to the original.

Solutions and agents used were as follows:

Ringer	Na^+	K^+	Ca^{2+}	Mg^{2+}	$Tris^+$	(in mM)
normal	80	4	7	5	5	
Na-free	0	4	8	5	85	(all in Cl^- form)

Agents

veratrine 5, 10, 15, 20, 30 µg/ml
tetraethylammonium (TEA) 30 mM (in Br^- form)
tetrodotoxin (TTX) 10 µM
Co^{2+} 15 mM (in Cl^- form)
Ni^{2+} 15 mM (in Cl^- form)

Veratrine, TTX and Tris were from Sigma, TEA was from Aldrich, all other compounds were from Reanal. The pH was set to 7.5. No osmolarity compensation was made.

RESULTS

Studies in current clamp

On the basis of previous experience (Erdélyi, unpublished), veratrine was applied at a concentration of 30 µg/ml and its effect was examined in current clamp mode. It soon turned out, however, that at this dose neurons soon go to the final state of veratrine effect and remain permanently depo-

larized. Thus, lower concentrations were applied, too (see Methods). It is noteworthy that, after some time, the activity of the neurons studied reached the same final state at any veratrine concentration and only the speed of development of effects was concentration dependent. In Fig. 1 the effects of 10 and 20 µg/ml veratrine on the neuronal activity and basic membrane parameters are demonstrated. According to chromatographic analyses (6) these concentrations are equivalent to 6 and 12 µM pure veratridine, respectively.

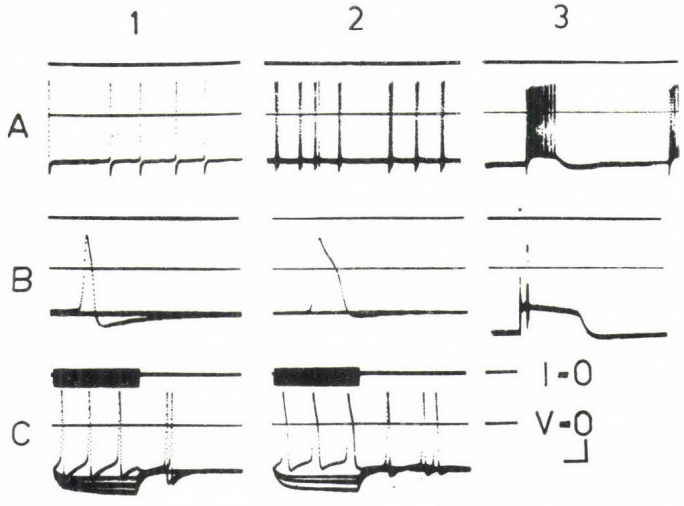

Figure 1. Effects of veratrine on the neuronal activity and basic membrane parameters.

1. Control. A: spontaneous activity. B: a single spike. C: effects of de- and hyperpolarizing current pulses from -1 to +3 nA.
2. As in 1, but recorded after 15 min of 10 µg/ml veratrine application. A: The activity turned into bursting and a small after-depolarization appeared following the bursts. B: the spike has been broadened and the negative afterpotential reduced in length and amplitude. C: action potentials became broader without appreciable change in resting potential, membrane resistance and time constant (as judged from the onset, steady level and decay of hyperpolarizations).
3. Further development of veratrine effect in time. Concentration was raised to 20 µg/ml in the 35th min. A: spontaneous PDS taken in the 55th, B: another one in the 135th min. The amplitude and length of plateaus increased with lapse of time.

Calibration: 20 mV voltage, 5 nA current for each part. Time: 1 s for 1/A, 20 ms for 1/B and 2/B, 100 ms for 1/C and 2/C, 5 s for others. Zero potential and current levels are indicated by continuous lines.

The neuron was a pacemaker one beating with some irregularity, its resting potential was about -45 mV (1/A). The spike, moderately wide (10 ms at

half amplitude) had a very little plateau and a considerable after-hyperpolarization (1/B). In the 15th min of veratrine action (column 2) the spike activity markedly changed (2/A), short bursts instead of single spikes occurred. The hyperpolarizing afterpotential decreased within the bursts and a weak after-depolarization appeared at the end of them. The membrane potential did not change. (Note different time scales between 1/A and 2/A.) Spike potentials also showed alterations (2/B): their width was more than twice the control value. The plateau was broadened and the decaying limb slowed down. Decrease of the after-hyperpolarization is clearly seen. Records in row C show the effects of current steps. Neither time course nor steady value of the potential changes caused by hyperpolarizing current steps were altered by veratrine. Values of membrane potential, resistance and time constant are summarized in Table 1.

Table 1. Membrane parameters of the neuron in Fig. 1 and the effect of veratrine

	resting potential (mV)	resistance (MOhm)	time constant (ms)
control	46	8.1	36
veratrinized	46	8.3	41

Further development of veratrine effect can be seen in column 3, Fig. 1. After 35 min the concentration was raised to 20 µg/ml. The small after-depolarization seen in 2/A grew to a typical paroxysmal depolarization shift by the 55th min (3/A). It was of moderate amplitude yet, as no spike inactivation occurred during its plateau phase. Only the first spikes showed some after-hyperpolarization. The PDS occurred and ceased spontaneously and had some negative afterpotential. A next PDS was triggered by a short current pulse in the 145th min (3/B). Its plateau was higher and more rectangular in shape than the previous one. Its decay had two phases: the plateau was slowly falling and this turned abruptly into a fast decay continuing in an after-hyperpolarization. Here, even the first spike is without negative afterpotential. The ones riding on the PDS seem to have it, but, as they do not reach the resting level, are not really negative. The burst-type activity induced by veratrine had always a time course like that described above. At the beginning, the bursts were short and their after-depolarization wave was small. With lapse of time this wave increased

in height and length. At first this produces a prolonged burst with spikes riding on the wave. Finally the wave amplitude exceeds spike inactivation level and a pure, square-shaped PDS appears, occasionally without any spike even at the beginning. Such a sequence is demonstrated also in Fig.3.

In accordance with findings of others (8, 10, 15), neurons were sometimes permanently depolarized by veratrine. This required more than half an hour to set in. The regenerative spike mechanism was not destroyed in these cells. Applying a constant hyperpolarizing current we could bring the membrane potential close to the normal resting level. With brief current pulses from here spikes and bursts or PDSs could be elicited. Fig. 2/E and F shows such records.

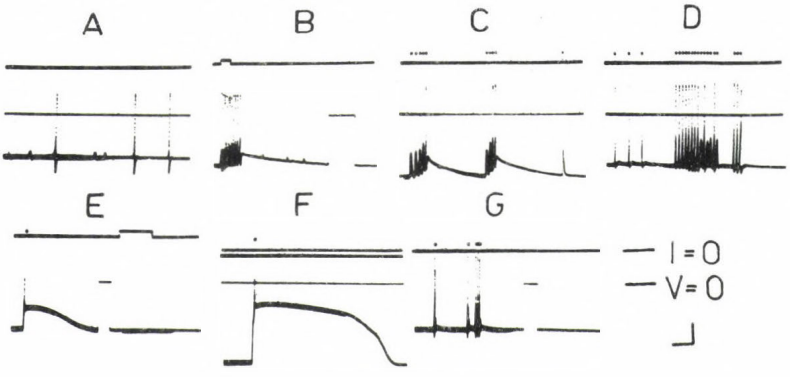

Figure 2. Development of veratrine action and its ionic dependence.
A: Control activity. B: Evoked burst after 4 min in 30 ug/ml veratrine. C: In Na-free Ringer (5 min), spikes are triggered only by stronger currents, bursting is absent and plateaus decay rapidly. D: 10 min later in Na-free Ringer, single spikes but no bursts and plateaus are evoked even by frequent trains. E: Returning to normal Ringer, the neuron got depolarized. It was kept at normal resting potential by continuous current injection and a PDS is triggered by a brief depolarization. F: Application of 15 mM Co^{2+}. The neuron still had to be kept hyperpolarized. A depolarizing pulse evoked a rudimentary spike and a huge, lengthy PDS. G: Again in Na-free Ringer. Spontaneous depolarization ceased. Full-sized spikes could be evoked but they did not trigger a PDS.
Calibration of voltage and current as in Fig. 1. Time: 1 s for A to D, 5 s for E, F, G. Zero levels indicated by continuous lines or brief jumps to that level (in B, E, G).

It is generally assumed that Veratrum alkaloids affect sodium channels. Using Na-free Ringer we tested if the action of veratrine is in relation to them in Helix neurons. Bursting activity was induced in several cells by veratrine and then Na was replaced by Tris in the perfusion fluid.

Bursts and PDSs seen under veratrine effect disappeared or were strongly reduced in Na-free Ringer, while spike potentials remained. Fig. 2 (A-D and G) and Fig. 3 (1/A, 2/C) show the effect of Na-removal. Preservation of spikes in Na-free Ringer means that they are carried by ions other than Na. This component (probably Ca-current) was apparently not affected by veratrine.

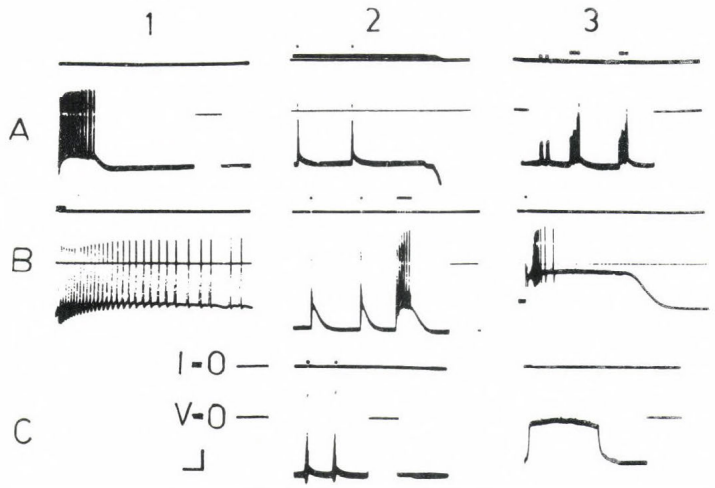

Figure 3. Ionic dependence of veratrine effect.
1. A: PDS from a neuron at the 20th min of 20 ug/ml veratrine action. B: Beginning of another PDS recorded with faster speed. Spikes on the plateau are characteristically broadened.
2. A: Effect of 15 mM Ni^{2+} at 16 min of application. The neuron got hyperpolarized and was kept at normal resting level by current injection. Further depolarizations (short jumps on the current line) elicit a weak spike and a minimal after-depolarization. B: Ni^{2+} washed out. Spike discharges and plateaus could be elicited by current pulses. No spontaneous activity. C: In Na-free Ringer only spikes could be evoked without plateaus.
3. A: Simultaneous effect of Na-deprivation and Ni^{2+}. Neither spikes nor depolarizing waves could be evoked even by impulse trains. B: Na concentration restored, a current pulse evoked a typical PDS with small spikes. C: Spontaneous late type PDS recorded in the wash out period.

Indication of zero levels as in Fig. 2, calibration of voltage and current as in Fig. 1. Time: 0.5 s for 1/B, 5 s for all others.

In order to elucidate the site of action of veratrine on the neuron membrane, it was of interest to know by what means the veratrine-induced current could be blocked. The current being Na-dependent, the first choice would have been TTX, but it has been published (8) that on snail neurons TTX did not block the slow current induced by veratridine. Therefore we

decided to test the effect of both TTX and some divalent cations known as Ca-channel blockers. In accordance with findings referred to above, TTX was not able to block the veratrine-current in our experiments. Fig. 5 shows that the current did not vanish or decrease but even went on growing. It is important to note that normal spikes of Helix neurons are also TTX resistant (11). Two divalent cations, Co^{2+} and Ni^{2+} were tested as possible blockers. Co^{2+} reduced or blocked the spikes but was largely ineffective in blocking the depolarization waves. Fig. 2 shows that in a medium with 15 mM Co^{2+} veratrine still can cause a PDS. Also steady depolarization continued, indicating that the non-inactivating part of the veratrine-current is also resistant to this concentration of Co^{2+}. Ni^{2+} is known to be a more potent blocker of TTX resistant inward current than Co^{2+}. In 15 mM concentration it greatly reduced both the spikes and the depolarizing waves triggered by them. The neuron in Fig. 3 went over into a deep hyperpolarization on application of Ni^{2+}. It had to be kept at a normal level by current injection; without that the potential went to more negative (part 2/A, hyperpolarization out of screen). Spikes evoked under Ni^{2+} effect were markedly reduced in height and after-depolarizations following them were short. This blocking effect was not significantly increased by simultaneous Na-removal (3/A in Fig. 3). Ni^{2+} was thus very effective in blocking both the normal and the veratrine-induced inward current.

Studies in voltage clamp

By means of single-electrode voltage clamp we analysed the I-V curves of the neurons in normal and veratrinized state. The first curve in Fig. 4 represents the control state of Helix neurons. A value close to the normal resting level was chosen as holding potential; here it was -50 mV. On hyperpolarization a purely ohmic leakage current developed. It was mostly small, correction for leakage was not usually necessary. On depolarization, the slow outward current could be seen. Due to the time dependence of this potassium-current, the curve had a hysteresis. Sharp distortions on its upstroke are spike artifacts from poorly clamped axonal area. Application of veratrine (15 µg/ml for 7 min) caused no major changes. The hysteresis moderately grew and the intersections of the curve (both at upstroke and downstroke) with the abscissa shifted to more positive direction. These phenomena indicate that a voltage-dependent inward current began to develop, and, on the other hand, the membrane without voltage clamp would have

been depolarized, probably due to a persistent inward current. To obtain the veratrine-induced current in pure form, voltage-dependent K-current was suppressed by TEA. To have a rapid onset, but slow development of the veratrine effect, its concentration was lowered to 10 µg/ml. The third curve shows how suppression of K-current unmasks the veratrine effect. To prevent a partial persistent inactivation of the veratrine-current the holding potential was raised to -80 mV. Now (fourth curve) the slow inward current was the only active one. It was first activated at -45 mV. Its maximum was at zero potential and inactivated fully, reaching +20 mV. The downstroke of the curve shows small if any inward current indicating that inactivation is removed slowly. This holds also for the persistent part of the inward current shown by the difference between the leakage lines (leftmost) of upstroke and downstroke.

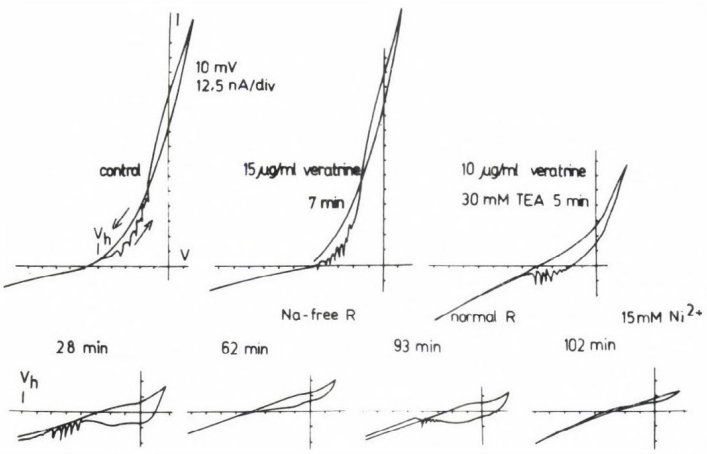

Figure 4. The effect of veratrine on the I-V curves alone and in combination with TEA. Records not corrected for leakage. Holding potential -50 mV for the first 3 curves, -80 mV for the others. Rate of ramp command: 20 mV/sec.
The control I-V curve was not much altered by 15 µg/ml veratrine in 7 min. On depression of the slow K-current by 30 mM TEA a slow inward current appears, rendering the curve N-shaped. The inward current disappears in Na-free Ringer or in 15 mM Ni^{2+}. A continuous shift of the resting membrane potential (where holding current is zero) towards 0 mV can be seen. Continuous time marks are for veratrine, other effects recorded when fully developed.

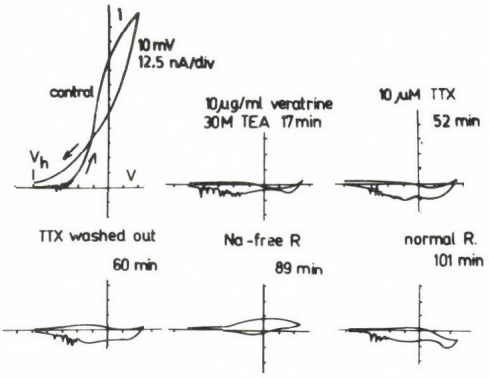

Figure 5. The veratrine-induced slow inward current is Na-dependent but resistant to TTX. Curves corrected for leakage (see Methods). Veratrine induced a negative slope conductance region on the I-V curve. The slow inward current remained also at a relatively high dose of TTX and became even stronger. In Na-free Ringer the current disappeared almost completely, on restoring Na concentration it reappeared. Holding potential is -50 everywhere. Time indication and other parameters as in Fig. 4.

In sodium free Ringer the veratrine-induced inward current disappeared also under these circumstances. In the fifth curve the hysteresis is greatly reduced due to its almost total absence. Returning to normal Ringer the current rapidly reappeared. During the experiment presented in Fig. 4 and several others, we found a continuous shift of the resting potential level in positive direction. The current caused by veratrine seemed to have a persistent part which slowly increases and makes the membrane depolarize. This explanation is supported by the fact that the shift was stopped when the inward current had no carrying ion or was blocked. The I-V curves taken under veratrine effect cross the abscissa usually twice, with positive slope at both points. This means that the neurons can be bistable. It was possible indeed to shift the membrane between the stable points, that is, to trigger or break a PDS.

Fig. 5 shows I-V curves from a different neuron treated with veratrine and TEA. After correction for leakage the net active currents are demonstrated. The slow rate of development of the veratrine-induced current can

be clearly seen. In the 17th min the inward current flows mostly between the holding level and 0 mV. At about the 60th min a considerable part of it is in the positive potential range and at 100 min it is hardly inactivated at the peak level of the ramp pulse, +20 mV. It looks like that, as time lapses, the sodium current induced by veratrine was not only activated at lower and lower potentials but it also remained activated at far positive levels. A change in the proportion between the time dependent and persistent component of the veratrine-induced current may underlie this phenomenon. This Figure also shows the sodium-dependence and TTX-resistance of the current (third and sixth curve). Neither the current itself nor its growth in time is affected by TTX. In Na-free Ringer practically no inward current remains. The small outward current usually seen (also here) was apparently insensitive to TEA at the concentration used, but it did not interfere with the properties of the veratrine-current studied by us.

DISCUSSION

The results presented here indicate that Veratrum alkaloids have a strong effect on the activity of Helix central neurons. The central issue is a novel ionic current not present in untreated cells. This current proved to be Na-dependent in accordance with observations on neural elements (1, 8, 10) and muscle fibres (3, 15). Veratrine induces this current most probably by affecting the channels of fast inward current. It has been demonstrated (2, 16) that veratridine attaches directly to the Na-channel and deeply alters its kinetics and voltage dependence. Channels affected open at lower depolarizations and inactivate very slowly, or, maybe, not at all. Thus a voltage-dependent slow inward current can flow which has been separated in our experiments. Its development takes a long time. It is supposed that veratridine binds mostly to open channels (5, 14). This may explain the broadening of the spikes within a burst.

The phenomena caused by veratrine or its purified alkaloids are similar to those caused by known epileptogenic substances. Veratrine can induce bursts, PDSs and N-shaped I-V curves like pentylenetetrazol on molluscan neurons or penicilline and aminopyridine on mammalian brain. An inward current with slow kinetics may underlie the action of known convulsants and veratrine. Thus it seems that veratrine or its alkaloids may help in constructing new experimental models of the epileptic state.

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DISCUSSION

AKAIKE, N.: What kind of mechanism have you in mind regarding the suppression of veratrine response by Na?

PAPP, A.: It has been demonstrated that veratrine (and its alkaloids) act on the channels of fast inward current. In case of muscle and nerve fibres these are sodium channels which can be blocked by TTX in intact or veratrinized state. In Helix neurons the first inward current is a mixed, Na-Ca one and it is sensitive to Ca-blockers, like Ni^{2+} but not to TTX. We suppose that veratrine affects the inward current channels also in Helix but the altered channels remain Na^{2+}-sensitive.

WINLOW, W.: 1. Are you aware that general anaesthetics such as halothane can produce paroxysmal depolarizing shifts in Lymnaea? (Girdlestone and Winlow, 1987). Could there be a common mode of action with veratrine?

2. Potassium channel blockers such as TEA and 4AP can also produce PDS (Holden, Winlow and Maydon, 1982). How does this fit in with your explanation of the actions of veratrine?

PAPP, A.: 1. We did not know anything about that. Veratrine is supposed - and on other preparations proved - to act on sodium channels, or other channels of fast inward current. If anaesthetics do the same, there may be a common mechanism.

2. I think that if a substance is potassium-blocker, it is not necessarily a convulsant. There can exist, however, "hidden faster" neurons, in which a slow inward current is covered by the slow outward current. A channel blocker can thus unmask the bursting character.

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ACTIVATION AND BLOCKADE OF GLUTAMATE-SENSITIVE ION CHANNELS

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Our knowledge concerning the mechanism whereby l-glutamate produces excitation of arthropod muscles and a range of vertebrates neurons has advanced following detailed investigation of the ion channels associated with glutamate receptors. Some glutamate receptors (ion channels) have kinetic and pharmacological properties which determine the functioning of their synapses.

During the investigation of postsynaptic potentials and currents induced by motor nerve stimulation or glutamate application in muscle cell of blowfly (Calliphora vicina) larvae we studied certain properties of these glutamate-activated channels (Magazanik et al., 1984). All experiments were performed on 'improved' preparation: the neighbouring cells were previously dissected (Antonov and Magazanik, 1984). It was found in experiments using voltage-clamp cells or under focal extracellular recording that the mean life-time of open channels was 5.8 $\pm$ 0.1 ms (n=4) under holding potential -65 mV. This value decreased following depolarization and increased following hyperpolarization (Fig. 1). The potential dependence of the open state time was moderate: for changing the mean life-time e-fold, the membrane potential level must be shifted to 329 $\pm$ 29 mV. The direction of this potential dependence of the open state time obviously differed from that found earlier in the experiments on locust or crustacean muscles where the hyperpolarization shortened the life-time of open channels (Anderson et al., 1978; Stettmeier et al., 1983a) (Fig. 2). The reversal potential of the synaptic currents approximated

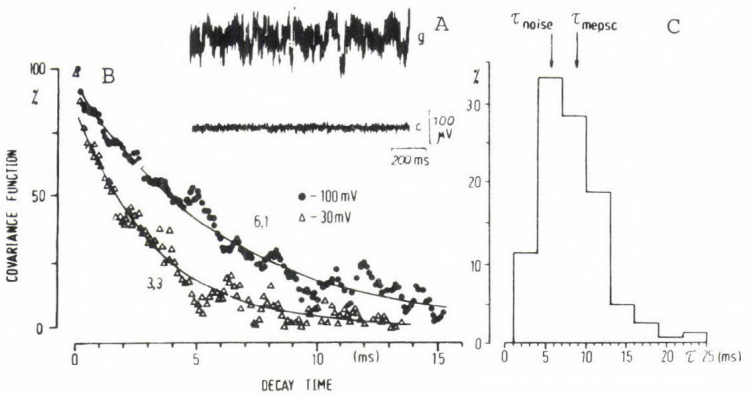

Fig. 1. Fluctuation of glutamate-induced current recorded in blowfly larval muscle. A - records of glutamate noise: g - during the application of 3 x 10^{-5} M glutamate, c - control. B - autocorrelation functions normalized by amplitude at MP= -100 mV (black circles) and MP= -30 mV (triangles); numbers - time constants in ms: solid lines - approximation by exponent. C - comparison of distribution of decay time constant of spontaneous postsynaptic currents (τ_{mEPSC}) and mean life-time (τ_{noise}) of open channels (by noise analysis).

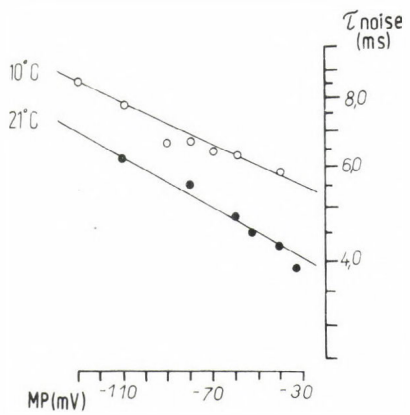

Fig. 2. Dependence of mean life-time of open channels (τ_{noise}, in ms) on voltage and temperature.

zero mV. The decay of miniature postsynaptic currents or the mean life-time of open synaptic channels determined by glutamate-induced fluctuation analysis was shortened by heating and prolonged by cooling of the preparation (Fig. 2). It was found that the decay of multiquantal responses evoked by nerve stimulation and of spontaneous monoquantal responses was exponential, starting from 60-70% of the maximal amplitude and the decay time constant exceeded the mean life-time of open channels (determined by glutamate-induced noise analysis) by 40% (Fig. 1C). This difference appears to be the result of asynchronous interaction of glutamate molecules and receptors in the course of the quantal response generation. The long-lasting application of l-glutamate (by addition to perfusing solution) induced the desensitization of glutamate receptors which expressed itself in the progressive decrease of quantal response amplitudes and the decline of input current induced by glutamate. Pretreatment of the preparation with 1×10^{-6} M concanavalin A during 30 min removed the phenomenon of desensitization to a great extent. The effect of concanavalin A on synaptic responses was moderate: the decay time constant shortened by 22% but potential dependence of decay remained constant (Fig. 3). The shortening was due to the effect of concanavalin A on the life-time of open channels since this effect was

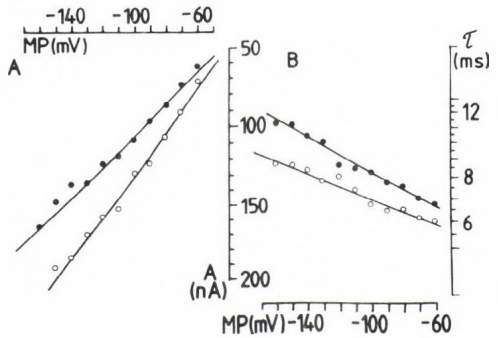

Fig. 3. Effect of concanavalin A on EPSC's: dependence of (A) current amplitude and (B) decay time constant (semilogarithmic scale) on MP level. Control - black circles; after 30 min treatment with 1×10^{-6} M concanavalin A (empty circles).

confirmed by glutamate-induced noise analysis. This differs greatly from results obtained earlier on locust preparation, where concanavalin A eliminated the potential dependence of life-time of open channels and in addition (in crustacean preparations) increased the time constant of decay responses (Mathers, 1981; Stettmeier et al., 1983b).

Potent and selective antagonists are very important for a detailed analysis of the function of glutamate-sensitive channels. We initially attempted to find drugs which block glutamate-sensitive channels from among the great variety of known blockers of acetylcholine-sensitive channels. One can reasonably suggest that two kinds of channels may have some common properties including pharmacological ones. But in experiments on the muscles of another fly, Drosophila melanogaster, it was shown that such cholinergic channel-blocking drugs as atropine, scopolamine, lidocaine, its derivative QX-222 were ineffective in the case of glutamate-sensitive channels (Magazanik and Vyskočil, 1979). The same negative results were obtained with hexamethonium on blowfly larval muscles. The synthesis of polymethylenebisonium derivatives with larger substituents allowed us to obtain drugs that combined the ability to block both kinds of channels: cholinergic in frog muscles and glutamatergic in fly muscles. The further screening resulted in creating several potent drugs which induced the blockade of glutamate-sensitive channels at rather low concentration (1×10^{-6} M) although their anticholinergic potency developed only at concentrations 50-100 times higher (Magazanik et al., 1984).

Another way to obtain potent selectively acting glutamate antagonists is to search for some neurotoxins in venoms of insectophage animals. It was found by Kawai et al. in Japan and by Tashmukhamedov et al. in the USSR that venoms of spiders from Araneidae family reduce the sensitivity of arthropod muscles and some vertebrate neurons to l-glutamate (Kawai et al., 1982; Tashmukhamedov et al., 1983). Usherwood and coworkers assumed that the main molecular component of these venoms, responsible for antiglutamate action, is a substance with a low molecular weight which is capable of blocking the glutamate-sensitive channels (Bateman et al., 1985).

In collaboration with colleagues from Shemjakin Institute of Bioorganic Chemistry (drs. E.V. Grishin and T.M. Volkova) we studied the biological activity of venom fractions and confirmed that one low molecular weight component readily inhibited the sensitivity to glutamate. This component, referred to as argiopin, was isolated and its molecular structure determined (Grishin et al., 1986; Magazanik et al., 1986, 1987) (Fig. 4).

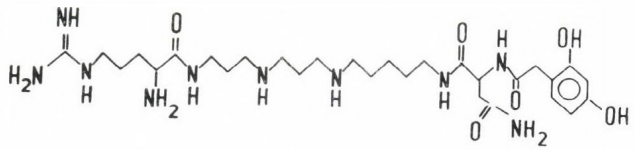

Fig. 4

Argiopin reduced to the same extent the amplitude of miniature EPSC's and multiquantal EPSC's evoked by motor nerve stimulation. This demonstrates that argiopin only blocks postsynaptically. In the frog preparation this postsynaptic effect was 36 times weaker, compared to that of blowfly larvae: 50% fall of EPSC amplitude was produced by argiopin at 4.4×10^{-7} M concentration in insect and 1.6×10^{-5} M in the frog preparation. From being exponential in control, the decay of EPSC's in both preparations became biphasic in the presence of argiopin. The initial and final segments of the decay curve could be approximated by two exponents: τ_1 (fast) and τ_2 (slow), correspondingly. These effects of argiopin were partly reversible, i.e. could be eliminated by prolonged washing (Fig. 5).

The form of the argiopin effect on glutamate and acetylcholine receptors appears similar to that of some blockers on postsynaptic ion channels. This allows the application of the sequential model of the open channel blockade (Adams, 1976) and to estimate both the rate constants of argiopin interaction with either open glutamate or acetylcholine channels (k_{+b} and k_{-b}) and dissociation constant (K_d). Under the same conditions (resting potential and room temperature) K_d of argiopin interaction with open glutamate channels was 36 times lower, com-

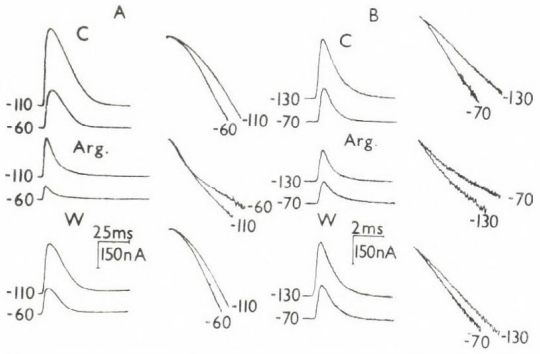

Fig. 5. Effects of argiopin on excitatory postsynaptic currents (EPSC) recorded in (A) blowfly larval muscles (4.4×10^{-7} M) and (B) frog muscle (4.4×10^{-5} M). Left - EPSC's on certain potential levels (indicated). Right - semilogarithmic plots of EPSC decay 100% to 5% of their amplitude. C - control; Arg. - after 30 min treatment with argiopin; W - after 60 min washing. Each curve is the result of averaging 16 responses.

pared to that of open acetylcholine channels ($6.7 \pm 1.5 \times 10^{-7}$ M, 6 experiments, and $2.4 \pm 0.3 \times 10^{-5}$ M, 5 experiments, respectively). It means that argiopin has preferential affinity to glutamate receptor.

The specific effect of argiopin on the open glutamate channels was confirmed by the analysis of current fluctuations. Argiopin produced not only a fall of integral glutamate-induced current but the changes in power density spectrum (Fig. 6). Control spectrum could be adequately described by a single Lorentzian, but in the presence of argiopin (4.4×10^{-7} M) it was fitted by two Lorentzians.

The analysis of current-voltage relationships of EPSC's revealed evidence for a dual mechanism of argiopin action. Besides the obvious blocking effect on the open state of glutamate-sensitive channels, argiopin may also interact with closed channels. These two kinds of interaction have the opposite potential dependence: hyperpolarization enhances the blocking effect of argiopin on the open state of channels but reduces that on closed ones. This conclusion was confirmed by analysis of EPSC's evoked by motor nerve stimulation at different time intervals after jump-like change in the membrane potential

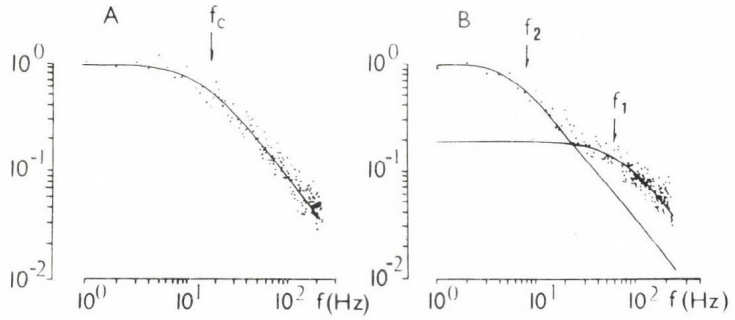

Fig. 6. Effect of argiopin on the mean life-time of the open glutamate channels. Normalized current spectral densities of glutamate-induced currents in blowfly larval muscle in control (A) and in the presence of 4.4×10^{-7} M argiopin (B). The continuous lines were obtained by least-squares fits of the points to a single Lorentzian (A) and to two Lorentzians (B). The arrows denote the out-off frequencies f_c = 14 Hz in control, f_1 = 48 Hz and f_2 = 7 Hz (corresponding time constants are 11 ms, 3.3 ms and 25 ms) after treatment by argiopin, MP = -60 mV, temp. 8°C.

level from -130 mV to -40 mV. In the presence of 4.4×10^{-7} M argiopin, the amplitude of EPSC's was found to be exponentially decreasing as the time interval between the potential jump and the EPSC increased. K_d of argiopin interaction with open or closed states of glutamate channel was quite similar at the resting potential -50 mV. But the fact that the potential dependence of interaction with these two states is in the opposite direction suggests that there are two different binding sites for argiopin on the glutamate receptor. One of them is functionally, and probably structurally, related to the open channel. The nature of the second site is far from clear.

It is very important to elucidate whether the potency of argiopin to block glutamate receptors in insects extends to that in vertebrates. For this purpose experiments were performed on isolated frog spinal cord since glutamate is the most likely transmitter in the amphibian sensory motor synapses. The purpose of the study was twofold: (1) to check by intracellular recording from the spinal motoneurons the potency of argiopin required to antagonize the glutamate- and aspartate-induced depolarization; (2) to examine by extracellular record-

ing the ability of argiopin to block synaptic transmission (Shupliakov et al., 1987; Antonov et al., 1987). The effect of argiopin on the amplitude of depolarization induced in motoneurons following short-lasting (30 sec) applications of glutamate or aspartate in a wide range of concentrations (3×10^{-5} - 1×10^{-3} M) were recorded, first in the basic solution and then in the presence of argiopin (2.3×10^{-8} - 5×10^{-7} M). The dose-response curves (DRC) were plotted (Fig. 7). The control

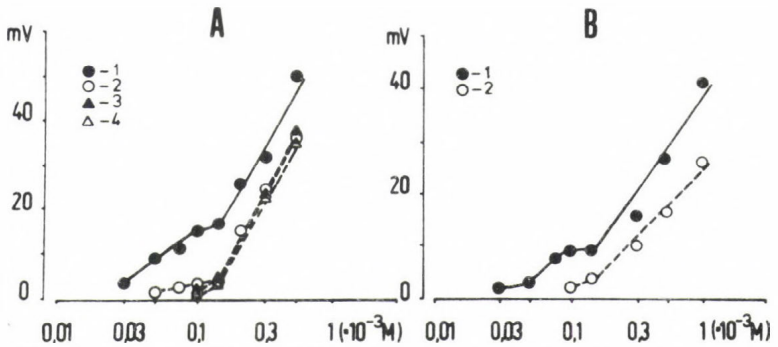

Fig. 7. Dose-response curves for glutamate and aspartate induced depolarization in motoneurons before and after application of argiopin. Abscissa: molar concentration of agonists (logarithmic scale); ordinate: motoneuron depolarization in mV. Circles: effect of glutamate; triangles: of aspartate. Control curves (black symbols); in the presence of 2.3×10^{-7} M argiopin (empty). Normal perfusing solution.

aspartate DRC was S-shaped with a linear part corresponding to 1.5×10^{-4} - 5×10^{-4} M. That of glutamate was more complicated in that it consisted of two parts. The initial one had a low amplitude and corresponded to the concentration range 1×10^{-5} - 1.5×10^{-4} M glutamate. The amplitude of responses did not exceed 15-20 mV. The final parts of the glutamate and aspartate DRC were similar. The glutamate DRC seems to be the sum of two components, one resembling the aspartate DRC. This assumption was confirmed by the argiopin action. Argiopin (2.5×10^{-7} M) did not affect the aspartate DRC, but induced a marked change in the glutamate DRC. In fact, the initial part of the curve

disappeared, while the final one coincided with DRC of aspartate. The ED_{50} of the argiopin effect was 7.5 $\pm$ 3.7 x 10^{-8} M (n=4). In most experiments the blocking effect of argiopin was not eliminated by prolonged (2 hours) washing.

The complicated course of glutamate DRC mentioned above could be due to the existence in frog motoneurons of at least two populations of receptors for excitatory amino acids. One activated either by glutamate or aspartate is responsible for the final part of the glutamate DRC and cannot be blocked by argiopin. The other is sensitive only to the activating action of glutamate, moreover to appreciably lower glutamate concentrations. These receptors are actually irreversibly blocked by argiopin. The question now is whether argiopin is capable of inhibiting the transmission through the glutamatergic synapses in the frog spinal cord. The electrical activity of ventral root was recorded as a response to stimulation of the corresponding dorsal root by single stimuli (Fig. 8). As a rule, the amplitude of the control root responses was 3-5 mV. In the presence of argiopin in the perfusing solution the ventral root response showed a concentration-dependent fall in amplitude.

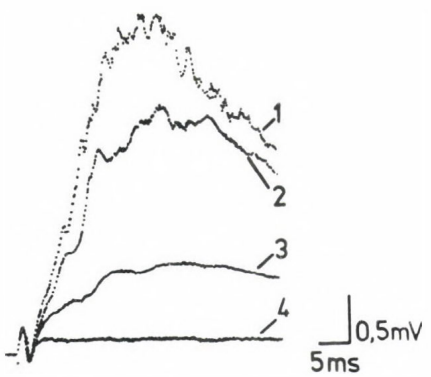

Fig. 8. The effect of argiopin (30 min perfusion) on the extracellularly recorded potentials evoked in the 9th ventral root in response to the stimulation of the 9th dorsal root. 1 - control; 2 and 3 - in the presence of argiopin 7.5 x 10^{-8} M and 2.3 x 10^{-7} M, respectively; 4 - total blockade of chemical transmission in the presence of calcium-free solution. Ten single responses have been averaged in each case.

The response decreased by 65-75% (30 min after the contact with 2.3×10^{-7} M argiopin). The interruption of stimulation for a short period induced a slow restoration of root response. This mode of action can be explained by the ability of argiopin to block the ion channels opened by glutamate. The restoration of transmission by washing was only partial and very slow (hours). Inhibition of synaptic transmission is evidently due to the postsynaptic blocking effect of argiopin on glutamate receptors. This supports the suggestion that some of these receptors (possibly of non-NMDA type) are involved in sensory-motor connections in the spinal cord. Our results show that the blowfly larval muscle preparation is quite adequate for investigation of glutamate receptors. It is very convenient particularly for the pharmacological study of newly synthesized drugs and naturally occurring neurotoxins. In spite of some peculiarities of insect glutamate receptors they share some common features with those of vertebrates. In particular, argiopin blocks effectively and selectively the glutamate-sensitive channels both in insects and vertebrate preparations. It allows us to use argiopin and in future its synthesized analogues as very important tools for the study of the function and chemical nature of glutamate receptors.

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DISCUSSION

HONEGGER, H.-W.: Do you know how the amino acid sequence of the Argiope-venom you investigated relates to the amino acid sequence of venoms of other Argiope species?

MAGAZANIK, L.G.: Recently the structure of the main toxic component isolated from Nephyla clavata venom was published by the Kawai group in Japan. This toxin of low molecular weight contains also the dihydroxyphenylacetic acid radical but there is no arginine residue.

LUKOWIAK, K.: What type of glutamate receptor does the toxin block in the spinal cord? NMDA, quisqualate or kainate?

MAGAZANIK, L.G.: Argiopin blocks receptors of quisqualate and kainate types but is unable to prevent the NMDA effects.

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NATURE OF FAST AND SLOW SYNAPTIC POTENTIALS IN INSECT MUSCLE

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Most insect muscle cells are innervated by two excitatory motor axons. Stimulation of the thickest fibre induced mainly fast twitches of cockroach leg muscles, but the stimulation of the thin fibre induced only slow contractions (Pringle, 1935). Later a concept appeared, by analogy to the vertebrate motor system, that insects have specialized fast and slow muscles innervated by two different types of motoneurons and their axons (Hoyle, 1974). In particular, investigation of postsynaptic responses evoked by motor nerve stimulation of body wall muscles of Calliphora erythrocephala larvae revealed, in each cell, two types of EPSP's differing in amplitude and duration: fast (larger) and slow (smaller), and with two types of contraction (Hardie, 1976; Hardie and Osborne, 1977). The difference in the time course of EPSP's may be due both to the difference in the cable properties of certain muscle cells and to the peculiarities of postsynaptic current generation. Under voltage clamp conditions fast and slow excitatory postsynaptic currents were found in the body wall muscle cells of Drosophila melanogaster larvae (Magazanik and Vyskočil, 1979). This fact could be interpreted as good evidence for the existence of two types of ion channels: both of them are sensitive to glutamate but differ in the mean life-times of their open state. We were forced to this conclusion since fast and slow postsynaptic currents recorded in frog m. ileofibularis (Magazanik et al., 1979; Fedorov et al., 1981, 1982; Miledi and Uchitel, 1981) and rat m. rectus inferior (Fedorov, 1987) originated from the

activation of ion channels and differed in mean life-time of open state. Differently localized sites sensitive to glutamate or aspartate were found on the surface on Musca domestica muscle cells (Irving and Miller, 1980a). This fact also seems to speak in favour of the existence of two different motor systems in insects. The authors assumed that l-glutamate is a specific agonist at the fast transmitter receptor, while l-aspartate is a specific agonist at the slow transmitter receptor (Irving and Miller, 1980b).

The facts mentioned above prompted us to more precise investigation of the postsynaptic ion currents in muscle cells of Calliphora vicina larvae (Antonov and Magazanik, 1984; Magazanik et al., 1984). The experiments were done on the muscle cells 6A and 7A (Crossley, 1965) of third instar larvae. The preparation was perfused with saline (normal): Na^+ - 172, K^+ - 2.5, Ca^{2+} - 0.7, Mg^{2+} - 6.0, Cl^- - 188, $H_2PO_4^-$ - 0.3, HCO_3^- - 0.6, sucrose - 42 (in mM), pH was 7.0. Intracellular recordings and current injections were made using 3 M KCl filled glass microelectrodes inserted near the centre of the muscle fibre. Glass micropipettes filled with normal saline and of a resistance of 1-2 megohm were used for extracellular recording of synaptic currents. The segmental nerves innervating 6A and 7A cells were stimulated via a suction electrode. Under voltage clamp conditions, two types of spontaneous and evoked postsynaptic currents were recorded: 'fast' and 'slow'. 'Slow' EPSC's were of smaller amplitude than 'fast' EPSC's. By changing the intensity or width of the stimulus we were able to elicit 'slow' and 'fast' evoked EPSC's independently (Fig. 1). Sometimes the currents had a composite time course being evidently the sum of 'fast' and 'slow' ones. But in any case the 'slow' currents were of smaller amplitude and had a longer latency.

Current-voltage relation and dependence of the EPSC time course on membrane potential level for 'fast' and 'slow' responses were compared and were found to be drastically different. The current-voltage relation of 'fast' EPSC's was linear as is typical for chemosensitive membranes (Figs 1 and 2). The decay of 'fast' EPSC's was exponential up to 60-70% of the

maximal amplitude. The decay time constant was 6.0 ± 0.2 ms (n=25), MP= -60 mV, t^{o}= 21^{o}C decreasing by depolarization and increasing by hyperpolarization. The dependence of the decay time constant on voltage was exponential (Fig. 2) $\tau(V) = \tau(0) \cdot \exp -V/H$ (Table 1).

The current-voltage relation for 'slow' EPSC's was not monotonic (Figs 1 and 3). When the holding potential changed from -30 to -90 mV the current amplitude increased. Further hyperpolarization induced the fall of current amplitude. At -120 to -140 mV the amplitude of 'slow' EPSC's became quite small. The decay of 'slow' EPSC's was 5-8 times longer than of 'fast' ones. The dependence of the decay time constant on membrane potential was much smaller and of the opposite sign (Fig. 3, Table 1).

Table 1. Parameters of 'fast' and 'slow' EPSC's (t^{o}= 21^{o}C)

Type of response	Rise time (ms)	$\tau(-60)$ (ms)	$\tau(0)$ (ms)	H (mV)
'Fast'	5.6 ± 0.2 (17)	6.0 ± 0.2 (25)	4.8 ± 0.3 (24)	+329 ± 29 (24)
'Slow'	28.4 ± 2.3 (16)	48.6 ± 0.4 (15)	65.4 ± 0.4 (15)	-448 ± 88 (15)

The focal extracellular recording gave a very surprising result: it was impossible to record any 'slow' or composite EPSC's. All spontaneous and evoked EPSC's were 'fast' (Fig. 4). Their voltage-current relation and dependence of decay time constant on the voltage were identical to those obtained in voltage clamp experiments. For this purpose three microelectrodes were used: two intracellular for voltage clamping and the third extracellular for focal recording.

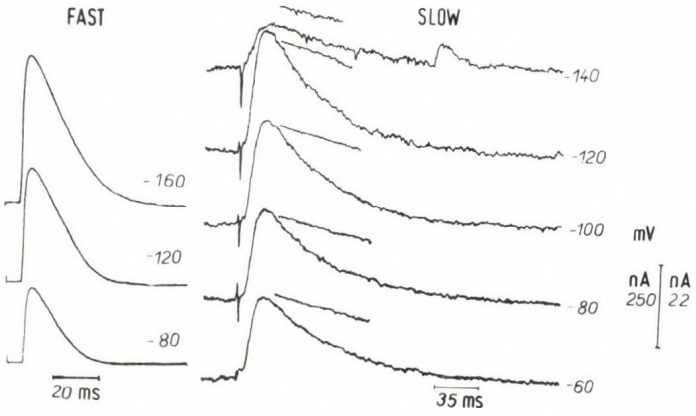

Fig. 1. 'Fast' and 'slow' postsynaptic currents evoked by nerve stimulation in 6A and 7A muscle cells of blowfly larvae at different levels of MP (denoted by figures). Each record obtained by averaging 16 responses.

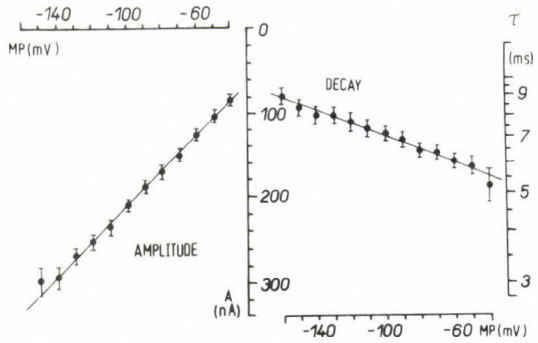

Fig. 2. Dependence of 'fast' EPSC amplitude and decay time constant on membrane potential level.

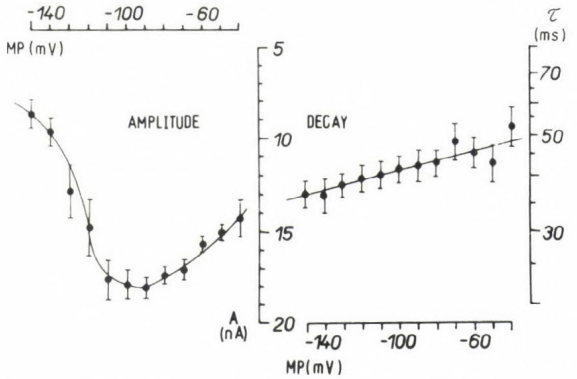

Fig. 3. Dependence of 'slow' EPSC amplitude and decay time constant on membrane potential level.

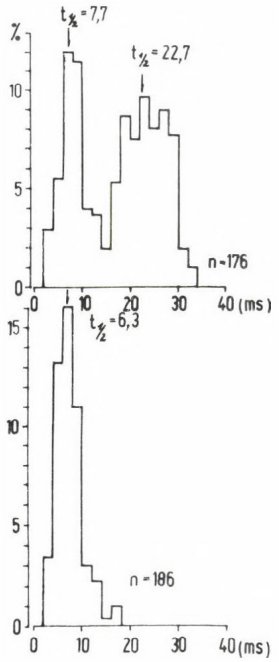

Fig. 4. Distributions of half-time of decay of spontaneous EPSC's recorded under voltage clamp (upper) and extracellularly (lower). Modal values denoted by arrows.

The discrepancy between results obtained under intracellular and extracellular recordings seemed strange until simultaneous recording from two neighbouring cells was performed. Two separate microelectrodes were inserted into neighbouring cells and spontaneous postsynaptic potentials were recorded simultaneously (Fig. 5). In many experiments they occurred synchronously but differed in amplitude and duration: being 'fast' and large in one cell, but 'slow' and small in the other. These synchronous spontaneous potentials recorded in neighbouring cells could be interpreted only as a result of the existence of effective intercellular electrical coupling (Antonov and Magazanik, 1984).

Verification of this suggestion was obtained in direct experiments. Rectangular current pulses were applied in one muscle cell and corresponding responses were recorded in this and neighbouring cells (Fig. 6). In all experiments a distinct

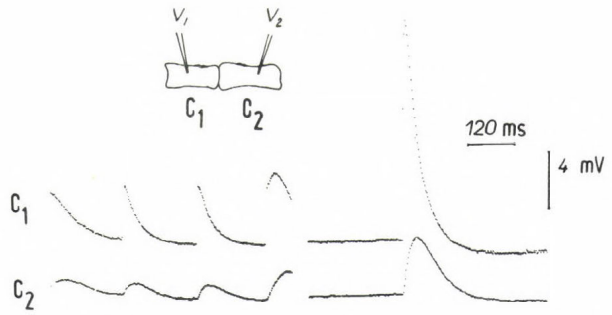

Fig. 5. Spontaneous 'giant' synaptic potentials recorded in two neighbouring muscle cells (C_1 and C_2).

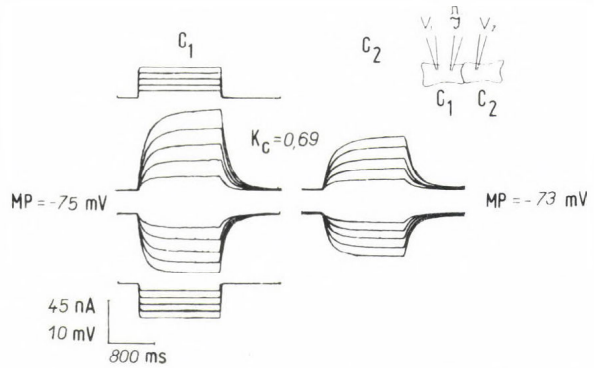

Fig. 6. Changes of membrane potentials recorded simultaneously in two neighbouring muscle cells (C_1 and C_2) induced by current injections into C_1. Insert: scheme of experiment. K_c - coupling coefficient.

electrical coupling between muscle cells was found. The ratio of response amplitudes recorded in these two cells (coupling coefficient) was 0.43 $\pm$ 0.06 (n=7).

An important peculiarity of electrical coupling in this preparation was a clear dependence on membrane potential (Fig. 7). The coupling coefficient remained relatively constant in the range of membrane potential between -30 and -70 mV and progressively decreased with hyperpolarization (Fig. 8). This fact is in accordance with the strange current-voltage relation of the 'slow' synaptic responses recorded intracellularly. It became

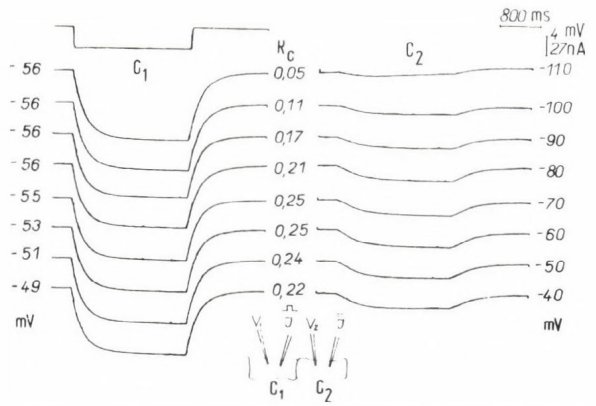

Fig. 7. Effect of membrane potential level on the changes of electrical coupling between two neighbouring cells. Insert: scheme of experiment. Left - potential changes induced by constant rectangular current injections in C_1. Right - responses recorded at different membrane potential levels (numbers) in C_2. K_C - numbers denoting the corresponding changes of coupling coefficient.

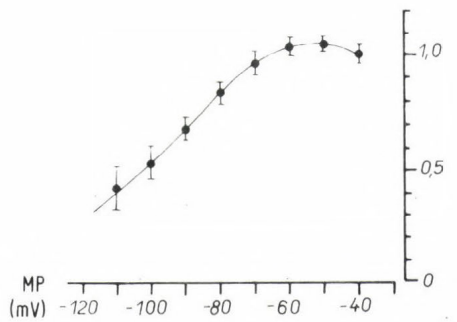

Fig. 8. Dependence of electrical coupling coefficient on the membrane potential levels (normalized to K_C at MP = -40 mV).

quite clear that these 'slow' responses originate from 'fast' synaptic currents, generated by one cell and transformed by electrical coupling in combination with the impedance of neighbouring cells. This causes a slowness of responses recorded from neighbouring cells and also decreases their amplitude.

Jan and Jan (1976) found Drosophila larvae muscle cells to be isopotential, such that the membrane potential could be altered uniformly by injecting current with an intracellular microelectrode. Thus, these cells seemed to be quite suitable for voltage clamping (Magazanik and Vyskočil, 1979), but the experiments mentioned above contradict these conclusions. The existence of electrical coupling makes the voltage clamp technique less effective due to a distinct leakage of injecting current to neighbouring cells. The anatomical features of muscle cell layer in the larval body wall allow us to improve this situation. These cells compose the longitudinal bands and each cell makes contact with two neighbouring ones. It was found that if these neighbouring cells were destroyed, the input resistance in the recorded cell increased during 30 min incubation in normal solution (Fig. 9, Table 2).

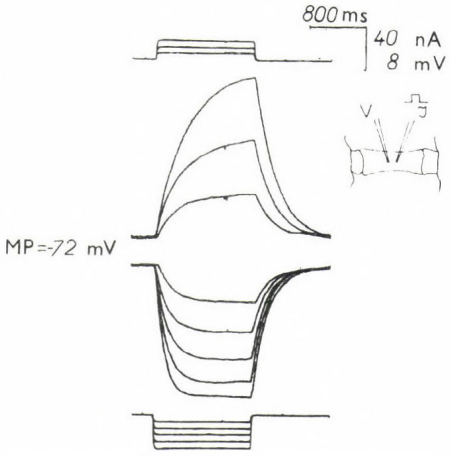

Fig. 9. Responses of isolated muscle cell to current injections (compare with Fig. 6).

It is very important to note that such procedure not only improved the conditions for voltage clamping but also removed 'slow' currents recorded before. Thus the discrepancy between the results obtained earlier under voltage clamp and focal extracellular recording completely disappeared. Only 'fast'

Table 2. Parameters of passive electrical properties of muscle cells

Cell	Intact preparation	Improved preparation		
	r_{in} (kOhm)	r_{in} (kOhm)	R_m (kOhm·cm^2)	C_m (µF/cm^2)
6A	232 ± 22 (5)	548 ± 95 (5)	9.1 ± 1.3	16.2 ± 0.8
7A	312 ± 53 (5)	740 ± 152 (5)	9.7 ± 1.0	16.0 ± 0.5

EPSC's could be recorded in this preparation. Since there are no differences in kinetics of activation of postsynaptic ion currents assumed earlier, the population of receptors and corresponding ion channels seems to be homogeneous. This supports our conclusion that 'slow' responses observed are artefacts originating from the electrical coupling between muscle cells.

The electrical coupling is probably due to the occurrence of highly permeable gap junctions between muscle cells. This was demonstrated with the help of the fluorescent dyes fluorescein and lucifer. Several minutes after intracellular injection of these dyes (iontophoretically through the microelectrode) into one cell, fluorescence could be clearly discerned in neighbouring cells due to spread of dye through the gap junctions between cells.

The rather high coupling coefficient observed at resting potential suggests that the electrical coupling between insect muscle cells may be significant for the functioning of the motor system. Under normal circumstances contractions of one cell may be followed by slower contractions of neighbouring cells. In addition, the degree of coupling is controlled by membrane potential level. Thus hyperpolarization induced by inhibitory nerves prevents not only the fast contractions directly induced by excitatory nerve but also the 'slow' potentials propagated along the muscle band since the efficacy of coupling is decreased by hyperpolarization. Evidently, the

coupling of muscle cells is dependent upon the intracellular calcium concentration similar to that found in other preparations (Deleze and Loewenstein, 1976; Rose and Loewenstein, 1976). It was noticed that the rate of input resistance increase, induced by destruction of neighbouring cells, depended on the calcium content in solution. This process was fast in the case of normal calcium concentration and became slower in low calcium solutions.

The general question arises as to whether these 'slow' synaptic potentials are common for all insects or whether the specific slow motor system really exists. Whatever the case, the possibility for effective electrical coupling between muscle cells as the mechanism of 'slow' responses must be taken into account.

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DISCUSSION

MOFFETT, S.: Do all muscle cells receive their own innervation?

ANTONOV, S.M.: Yes, all muscle cells receive such innervation as GA and JA cells and are innervated by two axons.

ROUBOS, E.W.: Is it possible to distinguish two types of muscle cells (slow and fast) with the electron microscope on the basis of, for instance, occurrence and distribution of thin and thick filaments?

ANTONOV, S.M.: Yes, it is possible. As was found in experiments made on cockroach and locust muscle cells 'fast' and 'slow' muscles are different in their morphology. But in case of Calliphora vicina body wall muscle fibres we cannot distinguish two types of cells. Most muscles are the same.

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MORPHOLOGY, SYNAPTIC CONNECTIONS AND NEUROTRANSMITTERS OF THE EFFERENT NEURONS OF THE CRAYFISH HINDGUT

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INTRODUCTION

The intestine of decapod crustaceans is under neurogenic control (Winlow and Laverack, 1972). The hindgut is mainly innervated from the last (6th) abdominal ganglion (Bullock and Horridge, 1965; Winlow and Laverack, 1972; Kondoh and Hisada, 1986). The light microscopic structure of the last abdominal ganglion has been analysed in the lobster (Winlow and Laverack, 1972c), and recently, the neuroanatomy of the ganglion and the distribution of motor and intersegmental interneurons has been described in detail in the crayfish Procambarus clarkii (Kondoh and Hisada, 1986).

Pharmacological and physiological experiments suggested three neurochemically different types of innervation (Florey, 1954, 1961 and unpublished experiments): cholinergic excitatory, non-cholinergic excitatory, and non-cholinergic inhibitory. The effect of catecholamines on the hindgut musculature has also been explored (Florey, 1954). A rich catecholaminergic innervation of the hindgut has been demonstrated in several crustacean species by the application of the Falck-Hillarp fluorescence technique (Elofsson et al., 1968; Aramant and Elofsson, 1976).

To further our knowledge of the innervation of the crustacean hindgut, we have examined the fine morphology and synaptic connections of the hindgut efferent neurons in the last ab-

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dominal ganglion of the crayfish Orconectes limosus after HRP and cobalt/nickel retrograde staining techniques, as well as the immunocytochemical localization of dopamine (DA) in the last abdominal ganglion-hindgut axis. The concentration of DA in the abdominal ganglia and in the hindgut was determined using a sensitive HPLC method.

MATERIAL AND METHODS

Adult male specimens of the crayfish Orconectes limosus were used for investigations. The animals were maintained in aquaria at 12°C in aerated water pumped in from Lake Constance.

Cobalt/nickel retrograde filling

Cut ends of the intestinal nerve (R7) were exposed to 4.5% $NiCl_2$ and/or 4.5% $CoCl_2$ (Quicke and Brace 1979) for one or two days at 7°C. For precipitation, 3 drops of saturated rubeanic acid in ethanol were added to 3 ml of 0.15 M Na-cacodylate buffer containing 3% sucrose for 15 minutes. The tissue was fixed in 4% formaldehyde (15 min), quickly dehydrated in 70% and 100% ethanol, cleared in methylsalicylate and finally mounted in Canada Balsam.

Retrograde HRP labelling

The cut end of the intestinal nerve was exposed to 12% HRP (Sigma, USA) for two days at 7°C. The last abdominal ganglion was fixed in 2.5% glutaraldehyde diluted in 0.1 M phosphate buffer for 4-5 hours at 4°C. After fixation, 80 µm frontal or sagittal sections were cut on a Sorvall TC-2 tissue chopper and processed for a peroxidase histochemical reaction, where 3,3-diaminobenzidine (Sigma, USA) served as chromogen and H_2O_2 as substrate. The sections were postfixed in 1% OsO_4, dehydrated and flat-embedded on slides in Araldite (Durcupan ACM, Fluka). The sections were analysed in the light microscope and then re-embedded for ultrathin sectioning and electron microscopy.

Immunocytochemistry

All tissue samples were fixed in 2-5% glutaraldehyde diluted in 0.1 M Na-cacodylate buffer, to which 1% Na-metabisulfite was added (Geffard et al., 1984a; Elekes et al., 1987). Three different techniques of peroxidase-antiperoxidase immunocytochemistry (Sternberger, 1979) were applied for the visualization of DA immunoreactivity: (i) wholemount technique for the intestinal nerve, (ii) cryostat section technique for the hindgut, and (iii) pre-embedding chopper technique (Elekes et al., 1987) for the last abdominal ganglion.

Anti-DA antibody (Geffard et al., 1984a,b) was applied in a dilution of 1:1000 or 1:2000 in phosphate buffered saline-Triton X-bovine serum albumin for ca. 20 h at $4^{\circ}C$. Specificity of the primary antibody was tested by incubating the corresponding tissue samples with the diluted antiserum exposed previously to an excess of immunogen (DA-glutaraldehyde-lysine complex, Geffard et al., 1984a,b).

High performance liquid chromatography (HPLC)

Tissue preparation

Isolated hindgut was frozen and powdered in liquid nitrogen, then homogenized in 0.4 M perchloric acid containing 0.1% EDTA and 0.05% potassium metabisulphide, and dihydroxybenzylamine as internal standard. After centrifugation, the catecholamines were isolated according to the method of Anton and Sayre (1962).

The individual abdominal ganglia were homogenized in 50 µl 0.25 M perchloric acid/0.1% EDTA, and after centrifugation aliquots of the clear supernatant were injected directly into the liquid-chromatograph.

Catecholamine determination

The catecholamines were analysed by ion pair-HPLC and electrochemical detection using a glassy carbon electrode. Isocratic separation was performed on a 4.6 x 250 mm C18 reverse phase column (Serva, Heidelberg, FRG), using a mobile phase of

100 mm citric acid, 50 mM sodium acetate, 0.0025% EDTA and 0.01% sodium octyl sulphate (pH 3).

Protein determination

The remaining pellets from the perchloric acid extracted tissues were digested in 100 µl (ganglia) or 500 µl (hindgut) 1 N NaOH at 95°C. After centrifugation, an aliquot of the supernatant was assayed for protein using the methods of Bradford (1976) and Gornall et al. (1949).

RESULTS

Distribution of the efferent neurons in the last abdominal ganglion following Co^{++}/Ni^{++} backfilling

The general pattern of innervation of the crayfish hindgut is shown in Fig. 1. After differential backfilling with Co^{++} and Ni^{++} through the intestinal nerve, three distinct groups of neurons appear in the posterior portion of the last abdominal

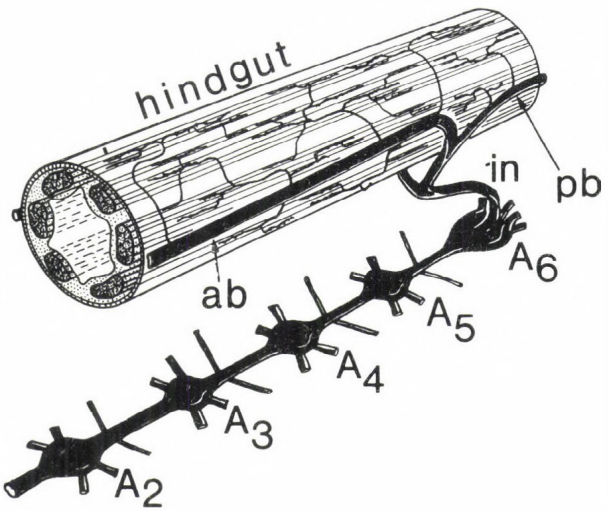


Fig. 1. Schematic drawing of the innervation of the crayfish hindgut through the last abdominal ganglion (A_6). in, intestinal nerve; ab, anterior branch; pb, posterior branch

ganglion, and three or four single, unpaired neurons in the anterior part (Fig. 2). The stained neurons are largely confined to the median plane of the ganglion. Altogether, 35 to 48 neurons are stained. About 20% of these neurons have axons in both the anterior and posterior branches of the intestinal nerve. Another ca. 20% have axons in the posterior branch only, and the majority of the neurons have axons exclusively in the anterior branches (Fig. 2).

In addition to the identification of the direction of peripheral projections, several major intraganglionic projections can be demonstrated and partially traced after Co/Ni filling. The most important findings are: (i) all of the neurons are unipolar and send their primary neurites into the ventromedian tract, or directly to the intestinal nerve. Lower order axon processes form loop-like projections in the sagittal plane (Fig. 2), and some of the neurons also form lateral projections; (ii) these axon processes do not give rise to extensive arborizations; (iii) many of the neurons send axon processes to the neural sheath of the ganglion where they ramify extensively.

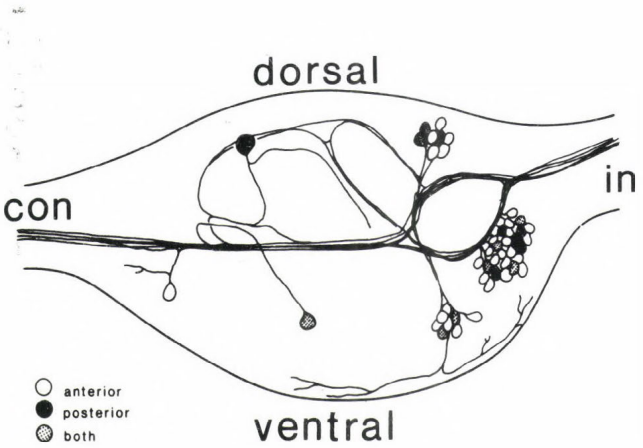


Fig. 2. Schematic representation of the distribution of the hindgut efferent neurons in the last abdominal ganglion after Co^{++}/Ni^{++} retrograde staining through the intestinal nerve (in). Sagittal view. con, connective

Fine morphology and synaptic connections of the efferent neurons following HRP backfilling

It is a prominent feature of several hindgut neurons that they send off secondary axon processes, some of them laterally, which form loop-like pathways while the primary neurite traverses the midline of the ganglion (Fig. 3). In frontal chopper sections, it is evident that these loop-like secondary axon processes return to the midline of the ganglion at the dorsomedian level, where they join the median bundle of primary neurites or enter the initial segment of the intestinal nerve root (Fig. 3). The primary neurites possess local arborizations of varicose fibres. These varicose fibres stem from fine collaterals rather than from the primary neurites. Fine varicose fibres can sometimes be observed close to the origin of the thick primary neurite. These local arborizations remain in the

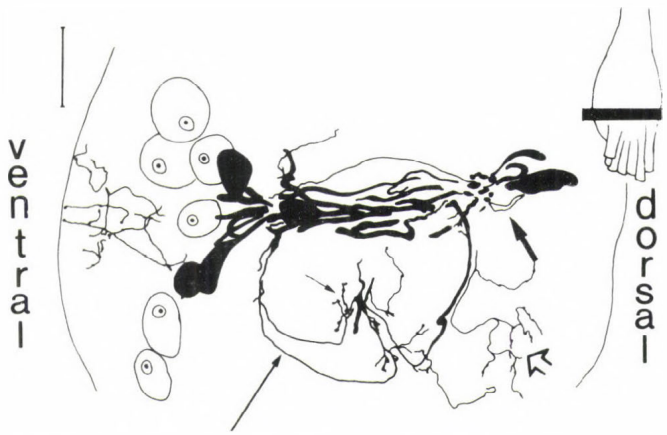


Fig. 3. Camera lucida tracing of HRP-labelled elements in an 80 µm frontal chopper section of the last abdominal ganglion at the level of the posterior ventral cell cluster (see also inset). HRP-labelled neurons (black) project dorsally to the "intraganglionic root" of the intestinal nerve (arrow) and arborize both in the neuropil (small arrow) and in the dorsal neural sheath (open arrow). Long arrow, HRP-labelled surface fibres in the ventral neural sheath. Bar represents 100 µm

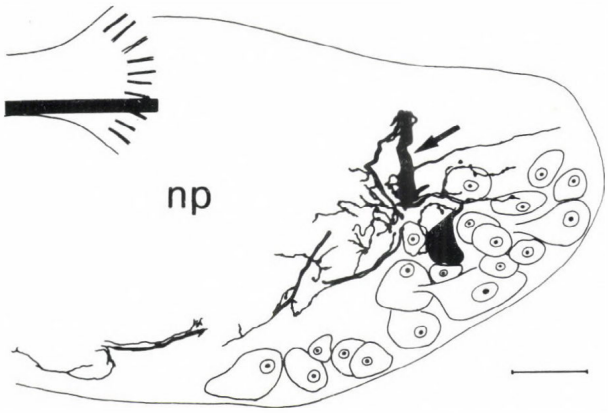
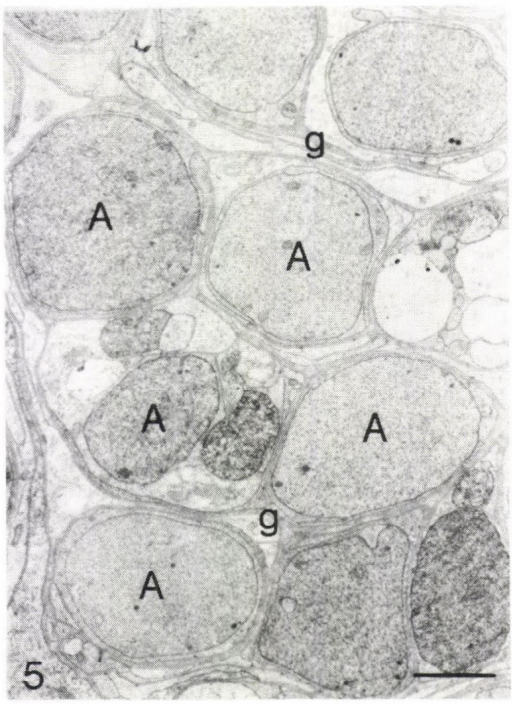


Fig. 4. Camera lucida tracing of an HRP-labelled hindgut efferent neuron (black) in a sagittal chopper section (see also inset). Most of the varicose fibres remain near the primary neurite (arrow) in and around the cortical layer of the ganglion. np, neuropil. Bar represents 100 µm

cortex and surround other cell bodies, while very fine processes invade the neighbouring neuropil (Fig. 4).

At the site of the local arborizations, HRP-labelled varicosities with mostly small (50-60 nm), agranular synaptic vesicles and a few large (ca. 100-120 nm) granular vesicles form specialized synaptic contacts with unlabelled postsynaptic elements. HRP-labelled axon profiles also occur in postsynaptic position to unlabelled terminals.

A surprising feature of the initial part of the intestinal nerve root is that it contains, in addition to the main axon processes, several thin collaterals and fine axon processes Figs 5, 6). In the intestinal nerve root, varicose processes and even larger axon processes containing vesicles and granules can be observed (Fig. 6). Two types of vesicles can be observed in these HRP-labelled axon profiles: large (ca. 100-120 nm) granular vesicles, and small (ca. 50-60 nm) agranular vesicles (Fig. 7). The large diameter axons are surrounded by glia processes (Fig. 5). In contrast, fine HRP-labelled axon profiles are relatively free from glia; the axons are in contact with each other. Here, both HRP-labelled and unlabelled axon



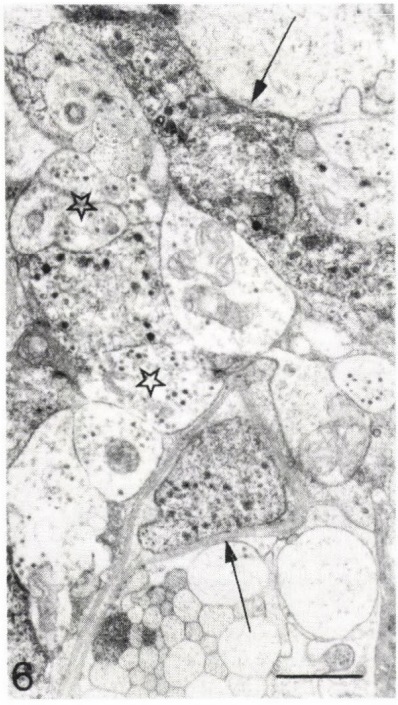
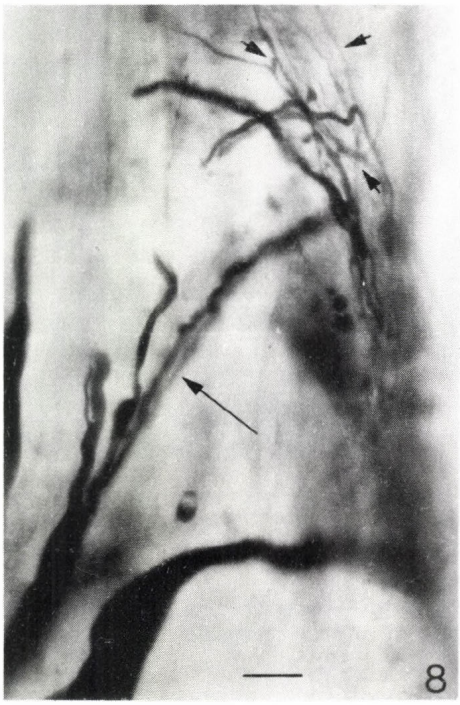


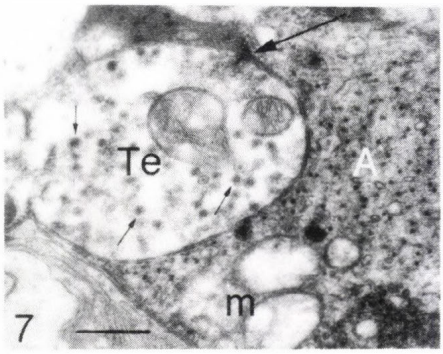
Fig. 5. Low magnification electron micrograph of the intestinal nerve root. HRP-labelled axon processes (A) are seen in cross section. Almost all of the labelled axons are individually surrounded by glia processes (g). Bar represents 2 µm

Fig. 6. Detail from the intestinal nerve root. HRP-labelled axon processes (arrows) and several vesicle-containing unlabelled varicosities (stars) are seen. Bar represents 1 µm

varicosities form specialized synaptic contacts. HRP-labelled profiles can be found in both pre- and postsynaptic positions (Fig. 7).

In addition to the central and peripheral (intestinal nerve) processes, HRP-labelled projections of the hindgut efferent neurons can also be traced to the neural sheath of the ganglia (Figs 8, 9). In the neural sheath there are numerous HRP-labelled varicosities which contain the same vesicle population as seen in the neuropil. The majority of the vesicles are small (50-60 nm) and agranular (Fig. 9). The varicose terminals are





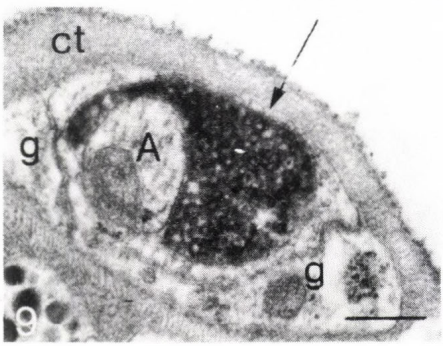


Fig. 7. A nerve terminal (Te) containing granular vesicles (small arrows) forms a synapse (arrow) on an HRP-labelled axon profile (A) in the intestinal nerve root. m, mitochondria. Bar represents 0.5 µm

Fig. 8. Axon processes (arrow) projecting from the ventromedial tract and arborizing (arrowheads) in the neural sheath of the most anterior part of the ganglion. Bar represents 25 µm

Fig. 9. A small HRP-labelled varicosity (arrow) filled with agranular and granular synaptic vesicles and an unlabelled axon profile (A) are surrounded by glia processes (g) and connective tissue elements (ct) in the neural sheath. Bar represents 0.5 µm

mostly embedded in connective tissue elements or are surrounded by glia processes (Fig. 9).

DA-immunoreactive elements in the last abdominal ganglion and hindgut

After applying the antibody raised against dopamine, stained neuronal elements can be observed in the last abdominal gangli-

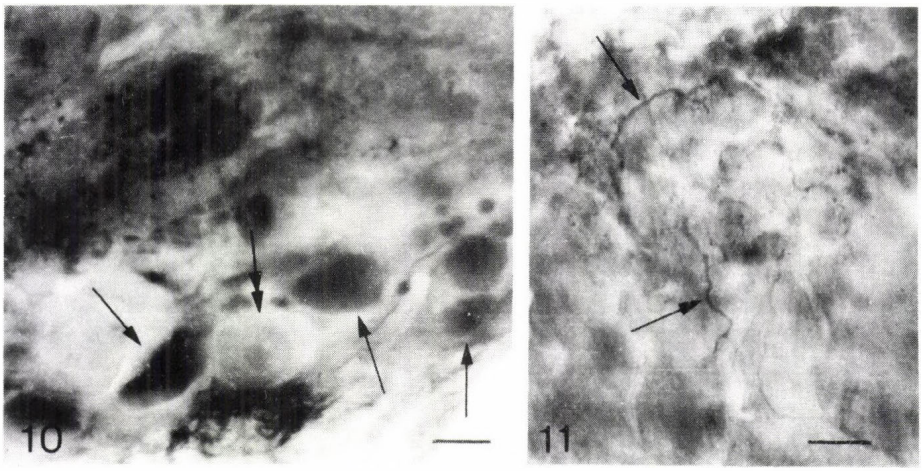


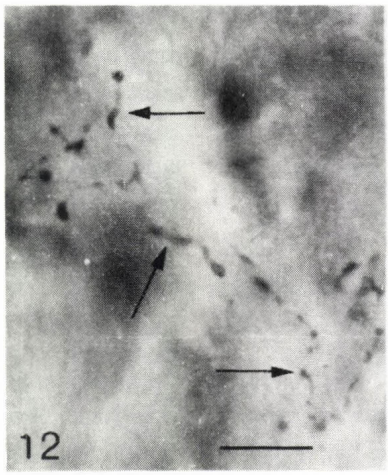
Fig. 10. DA-immunoreactive cell bodies (arrow) in the cortical layer of the ventral surface of the ganglion. Frontal chopper section. Double arrow, unlabelled cell body. Bar represents 20 µm

Fig. 11. Varicose arborization of a DA-immunoreactive axon (arrows) in the neuropil. Frontal chopper section. Bar represents 40 µm

on, in the intestinal nerve and in the hindgut musculature (Figs 10-15).

In the last abdominal ganglion, DA immunoreactivity (DAi) is seen in neuronal cell bodies (Fig. 10) and axon processes (Figs 11, 12). DA neuronal somata are found only in the cortical layer of the ventral surface; their number is small. No DAi can be unequivocally demonstrated in any of the hindgut efferent neurons labelled with Co/Ni or with HRP. In the neuropil of the ganglion, several thick DAi axon processes and rich varicose arborizations occur (Figs 11, 12): some of the non-branching, thick DAi processes exhibit a lateral pathway similar to that seen after retrograde HRP-labelling of hindgut neurons.

When anti-DA immunocytochemistry is applied to wholemount preparations of the intestinal nerve, four or five thick DAi axon processes can be observed (Fig. 13), which have a diameter of several µm and run mostly in a small, closely packed bundle. Some single DAi axons cross from one side of the intestinal nerve to the other.



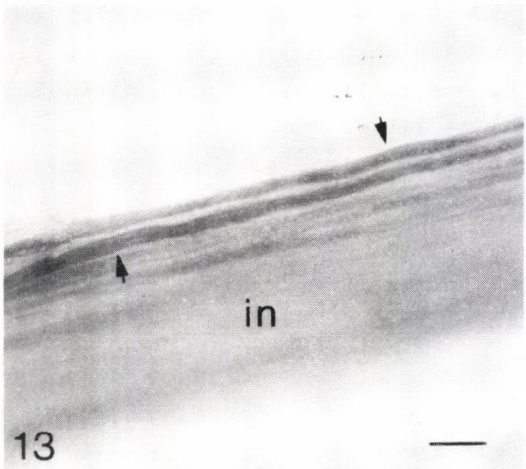
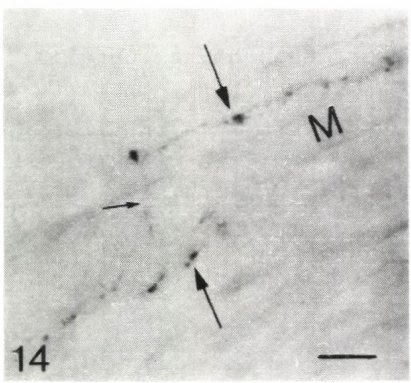
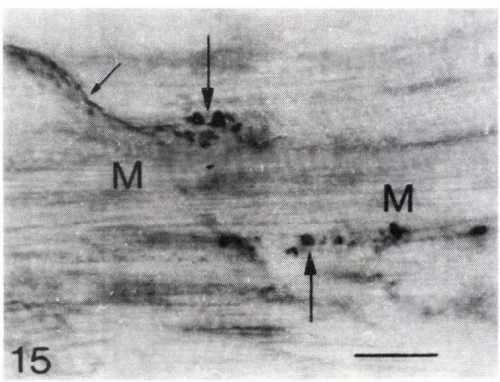


Fig. 12. High magnification light micrograph of DA-immunoreactive varicose fibres (arrows) in the neuropil. Frontal chopper section. Bar represents 25 µm

Fig. 13. DA-immunoreactive axons (arrowheads) in the intestinal nerve (in). Wholemount preparation. Bar represents 20 µm





Figs 14, 15. Cryostat sections of DA-immunoreactive varicose (arrowheads) fibres in the hindgut musculature. Varicosities are lined up along the muscle fibres (M). Nonvaricose axon segments (small arrows) cross over also several muscle fibres. Bars represent 20 µm

Several shorter processes of DAi varicose fibres are seen in cryostat sections of the hindgut, running mostly parallel to the muscle fibres. Smooth segments occasionally cross several muscle fibres and then continue along another muscle as a varicose axon (Figs 14, 15), much like a typical multiterminal innervation.

HPLC determination of DA in the abdominal ganglia and hindgut

The DA concentration of the abdominal ganglia ranges from zero to 4.4 pmole per ganglion. DA content is highest in the first and second ganglia (around 3 pmole) and decreases from the third to the last (6th) ganglion (Table 1). The last abdominal ganglion contains considerably less than any of the others. The protein content per ganglion does not show any apparent correlation with DA concentration, and when expressed as pmoles of DA/mg protein, the values vary greatly.

In the hindgut, DA has been found in similar quantities as in the ganglia, but the concentration relative to protein is about one order of magnitude lower than in the ganglia.

Table 1. Dopamine content of the different abdominal ganglia in Orconectes limosus

Abdominal ganglion	pmole/ganglion (± SD)	pmole/mg protein (± SD)
1	2.80±0.99 (N=7)	97.7±36.4 (N=6)
2	3.02±0.27 (N=6)	96.9±31.8 (N=5)
3	1.85±0.23 (N=7)*	73.1±24.2 (N=6)
4	1.31±0.23 (N=7)*	55.6±16.3 (N=6)**
5	1.14±0.41 (N=7)*	50.0±11.8 (N=6)
6	0.79±0.21 (N=5)*	22.9± 7.4 (N=4)**

* Significantly different (t-test, $p<0.02$).

** Significantly different (t-test, $p<0.005$).

DISCUSSION

The hindgut efferent neurons of the last abdominal ganglion of the crayfish Orconectes limosus are rather uniform in appearance. Without exception, they are unipolar (or pseudo-unipolar) and are characterized by two important features: (i) lack of well developed axonal arborizations within the ganglionic neuropil, and (ii) projection of lower order axon processes with profuse branching in the neural sheath of the ganglion. This neuroanatomy in entirely different from that of somatic motoneurons like those of the uropod closer muscle in the last abdominal ganglion of the crayfish Procambarus clarkii (Kondoh et al., 1987). The uropod closer motoneuron possesses a well developed dendritic arborization, but never projects to the neural sheath. This difference in axonal arborization might reflect functional differences between motoneurons innervating somatic muscle fibres and those efferent neurons which regulate non-somatic (visceral) musculature. The number of synaptic contacts established by the hindgut efferent neurons in the neuropil of the last abdominal ganglion is very small. Their terminals are both pre- and postsynaptic. This indicates that the hindgut efferents, even if only to a very limited degree, are involved in intraganglionic integrative processes, as are other crustacean motoneurons (King, 1976). A peculiar feature of the hindgut efferents is their axonal arborization in the intestinal nerve root where they also constitute both pre- and postsynaptic elements. This site of synaptic interaction may indicate an additional level of modulation of the efferent information sent to the hindgut musculature. In addition, it implies the involvement of the hindgut efferent neurons in the synaptic modulation of other peripheral processes, possibly even of afferent activity.

Synaptic interaction also occurs among identified central neurons in the intestinal nerve of the snail Helix pomatia (Elekes et al., 1985).

Another site of axonal projections of the hindgut efferent neurons is the neural sheath of the last abdominal ganglion. Here, varicose fibres may release their transmitter directly

into the abdominal blood sinus and/or into the blood vessels, thus participating in the neurohumoral modulation of peripheral processes. The presence of a network of varicose nerve terminals in the ganglionic sheath has previously been described in other crustaceans (Beltz and Kravitz, 1983, 1987) and in other arthropods (Nässel and Elekes, 1985; Barber, 1983).

For the efferent neurons innervating the hindgut and located in the last abdominal ganglion of the lobster, it has been shown immunocytochemically that some (the posterior median cell cluster) are proctolinergic. Those of the crayfish are not (Siwicki and Bishop, 1986), nor do they contain serotonin (Elekes and Florey, unpublished observation). In our present study, anti-DA antibody also failed to stain surface fibres or the somata of specific hindgut efferent neurons previously identified by retrograde labelling. In contrast, DA immunocytochemistry demonstrated varicose fibres in the neuropil of the ganglion, revealed immunoreactive axon processes in the intestinal nerve, and furnished evidence for the presence of DA-containing varicose fibres in the hindgut musculature. A catecholaminergic innervation of the crayfish hindgut has previously been demonstrated both pharmacologically (Florey, 1954), fluorescence histochemically (Elofsson et al., 1968) and ultrastructurally (Elofsson et al., 1978). The relatively slight immunolabelling of neuronal somata is difficult to explain, especially in view of the presence of DA-immunoreactive axon processes in the intestinal nerve and in the hindgut musculature. It is possible that the corresponding cell bodies do not contain a sufficient quantity of DA to be stained by the antibody or that only the single hindgut efferent neurons located in the first five abdominal ganglia are DAergic. These ganglia were not processed for anti-DA immunocytochemistry, but have been shown by HPLC measurements to contain DA. The lack of DA immunoreactivity in the surface fibres of the hindgut efferents in the neural sheath suggests two possibilities: (i) the hindgut efferent neurons are not homogeneous in their transmitter content; (ii) some of the hindgut efferent neurons, even the DAergic ones, may contain more than one transmitter substance which are released at different parts of the neuron. At the

ultrastructural level, at least two different types of vesicles (small agranular and large granular) can be distinguished in the synaptic terminals and axon varicosities of the hindgut efferent neurons. The presence of more than one transmitter in nerve cells of the ventral nerve cord of the lobster has most recently been demonstrated immunocytochemically (Siwicki et al., 1987). Some of these project both to the central neuropil of the ganglia and to the neural sheath (Beltz and Kravitz, 1987).

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DISCUSSION

PENZLIN, H.: What about the occurrence of neuropeptides in the crayfish hindgut? You did not mention them.

ELEKES, K.: Proctolin (and possibly other neuropeptides) would also be a good candidate for the hindgut efferent neurons in the 6th abdominal ganglia. Siwicki and co-workers have recently investigated the distribution of proctolin-immunoreactive neurons in the nervous system of lobster and crayfish. Meanwhile they have found the posterior median cell cluster to be proctolin-immunoreactive in the lobster, it was immunonegative in the crayfish. We did not find either proctolin-immunostaining for the hindgut efferent in the crayfish Orconectes limosus.

ROUBOS, E.W.: You have indicated four types of release sites. Is there direct evidence for the release of neurochemical messengers (e.g. peptides) from these sites, for instance the presence of exocytosis of granule contents?

ELEKES, K.: An additional (fifth) function for these hindgut efferents cannot, of course, be excluded: modulatory role through non-synaptic release in the last abdominal ganglion. However, we did not look systematically for exocytotic phenomena in the course of our EM analysis and up to now we have not found it.

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IMMUNOCYTOCHEMISTRY OF PUTATIVE NEUROACTIVE SUBSTANCES IN THE INSECT BRAIN

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INTRODUCTION

Chemical transmission in insects has been studied quite extensively in the peripheral nervous system. Preparations like salivary glands, heart muscle, visceral and skeletal muscles are useful both for studies of neuropharmacology of identified neurons innervating these tissues and neurohormonal actions[16,22,29,39,40,49]. In contrast little is known about chemical transmission in the central nervous system (CNS) of insects. It appears as if some well defined circuits or pathways need to be described that can be used for neuropharmacological studies of the insect CNS. Some attempts have been made in the visual system of flies at the photoreceptor synapses[17,18] and at the level of motion sensitive units[7]. To proceed further we need to investigate the neuronal distribution of neuroactive substances (transmitters and modulators) in more detail and to select appropriate chemically defined pathways for analysis. Immunocytochemistry has provided one convenient way of mapping the distribution, projections and connectivities of neurons using different neuroactive substances. Extensive immunocytochemical identification of putative neuroactive substances has been performed in the nervous system of different insects but few of these have provided detailed anatomical descriptions of neurons and their pathways. The present paper summarizes the immunocytochemical findings on putative neuroactive substances and outlines some chemical pathways in the brain and optic lobes that may be suitable for neuropharmacological/physiological studies. It should be noted at the outset that due to limited space many of the original literature references are omitted and are replaced by recent reviews.

SURVEY OF SYSTEMS USING DIFFERENT PUTATIVE NEUROACTIVE SUBSTANCES IN THE INSECT BRAIN

A large number of the neuroactive substances known from the vertebrate brain have also been identified or indicated in the insect brain. Among the peptides some seem to be present in forms similar but not identical to substances identified in the vertebrate brain,others may be

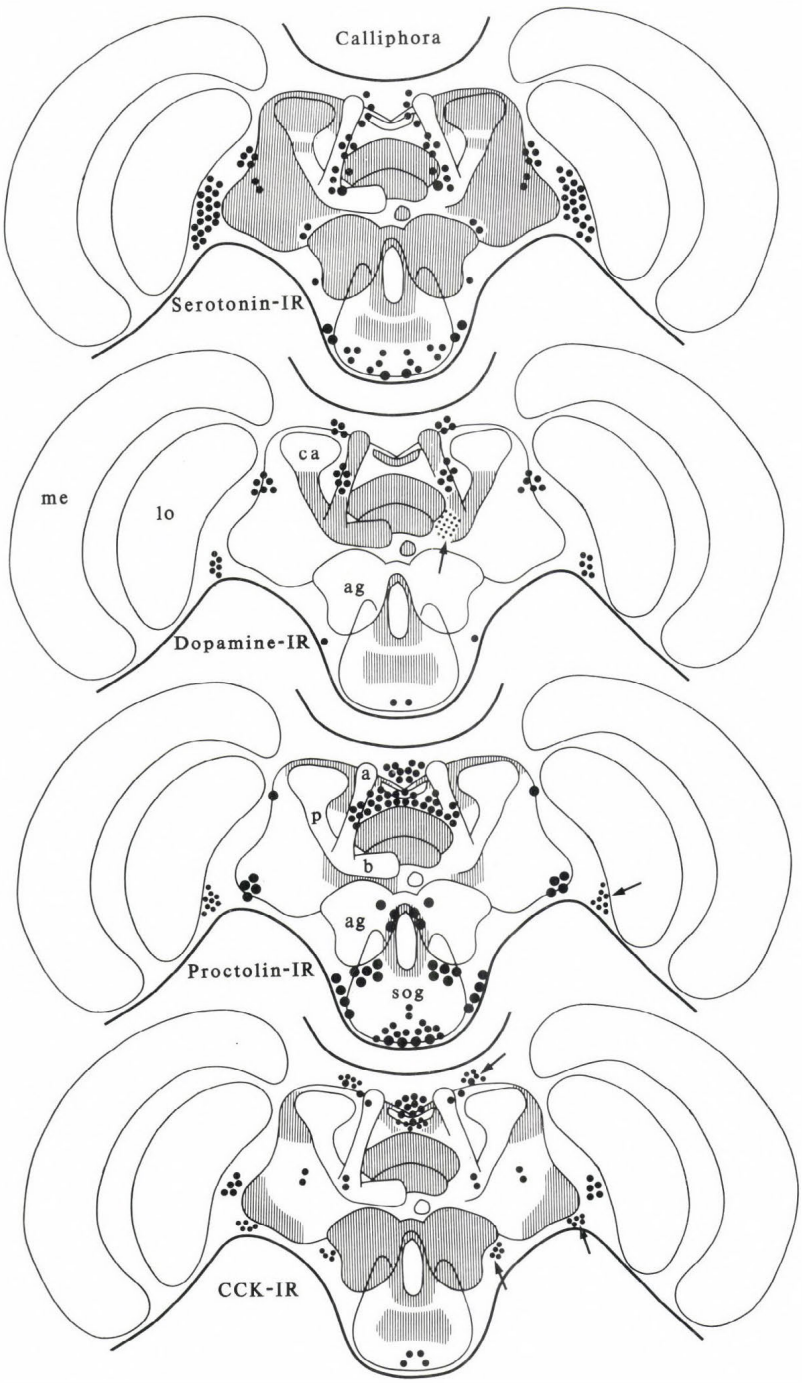
more specific to insects. Based on different assay methods the following putative neuroactive substances of "classical type" have been indicated in the insect brain: acetylcholine, the biogenic monoamines dopamine, noradrenaline, octopamine, serotonin, histamine, and the amino acids GABA, glutamate and taurine[9,17,18,22,25,29,30,51]. A large number of neuropeptides have also been indicated by immunocytochemistry. Only a few have, however, been isolated and sequenced: e. g. adipokinetic hormones (AKH) and the related myoactive substances MI and MII, proctolin, an inhibitory myoactive peptide leucomyosuppressin and the gastrin/CCK-like leucosulfakinin I and II [4,8,20,28,39,40,44]. Some further peptides have been partly characterized: prothoracicotropic hormone (PTTH) resembling the human insulin A-chain, a peptide (involved in insect melanization) similar to insulin-like growth factor II, a cardioacceleratory peptide (CC-2) resembling vertebrate glucagon and another called neurohormone D resembling AKH, a pancreatic polypeptide-like substance and substance(s) resembling enkephalin[2,10,15,28,40].

In the following a summary is presented of the general distribution of neurons and neural systems reacting with antisera to GABA, serotonin, dopamine, proctolin, FMRFamide and CCK. Emphasis is put on results from the flies *Calliphora* and *Drosophila*. In a separate section the optic lobes are presented. Electron microscopic immunocytochemistry, performed for serotonergic and peptidergic neurons[32,35,36,38], will be ignored in this account.

GABA. As shown in the elegant study of the moth *Manduca sexta* [21] antisera to GABA label very large numbers of brain neurons (ca. 40,000 in the brain and optic lobes). Most centers in *Manduca* as well as the honey bee[43] and blowfly[27,30] contain GABA-immunoreactive (GABA-IR) processes. Since GABA may be the major inhibitory substance in insects[46] it is not surprising that GABA-IR circuits and pathways are so ubiquitous in the brain. In the brain most GABA-IR neurons are interneurons of different types. These interneurons form local circuits or projection pathways. The inhibitory function of GABA can hence be studied at many different levels in many different systems. Few identifiable GABA-IR neurons have been described in the brain that may be analyzed physiologically (see, however, optic lobe section). Similarly it appears that putatively cholinergic neurons are numerous and widespread in the insect brain (based on studies with antisera to choline acetyltransferase[5]).

→

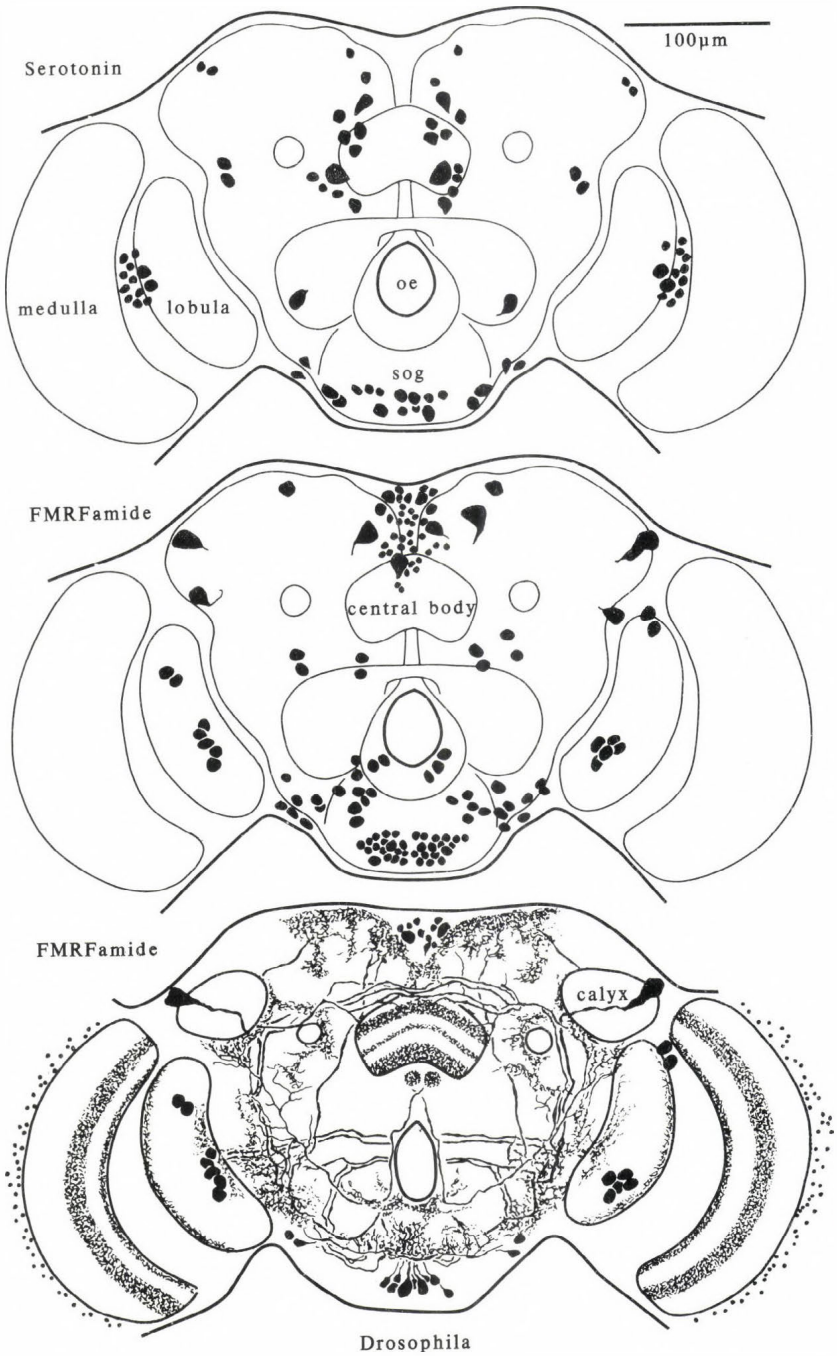
Fig. 1. Schematic diagrams of *Calliphora* brain (frontal view) with accurate numbers of immunoreactive cell bodies (filled circles) and neuropil processes (stippled). Immunoreactivity in the optic lobes is omitted. Abbreviations: me= medulla; lo= lobula; ag= antennal lobes; ca= calyx, p= peduncle; a= alfa-lobe; b= beta-lobe; sog= suboesophageal ganglion. Arrows indicate groups of cell bodies where accurate numbers are not shown. Altered from Refs. 29, 34 (Nässel, Johansson, Klemm, Steinbusch, in prep.).


Calliphora
Serotonin-IR
me
lo
ca
ag
Dopamine-IR
a
p
b
ag
Proctolin-IR
sog
CCK-IR

Serotonin (5-HT). 5-HT-immunoreactive (5-HT-IR) neurons have been mapped in several insects species[29,30] and their distribution can be generalized as follows. Very few neurons (ca. 100 in *Calliphora* and *Drosophila*; i. e. 0.1% of total neurons) display 5-HT-IR, but most of the neuropil areas (centers) of the brain are innervated by their processes (Figs. 1,2). Most of the 5-HT-IR neurons, hence, form very extensive processes invading large volumes of neuropil. The majority of the 5-HT-IR neurons connect several distinct neuropils.The neurons are distributed in bilateral pairs or clusters and many form bilateral connections innervating the same centers on both sides of the brain. In the brain all neurons, except four, appear to be interneurons. These four neurons (in the suboesophageal ganglion) are efferents with extensive neurosecretory terminals in the neural sheath of cranial nerves and the thoracico-abdominal ganglia[32]. Many of the 5-HT-IR circuits in the brain may be involved in modulation of neurotransmission in large volumes of neuropil. Released 5-HT may modulate responsiveness of neurons, e.g. in adaptation, arousal and learning. Further analysis of the physiological and behavioral role of 5-HT may be carried out by means of pharmacological lesioning experiments and use of *Drosophila* mutants deficient in serotonin metabolism[26,45]. Several 5-HT-IR neurons are individually identifiable and accessible for intracellular recordings.

Dopamine (DA). The morphology of dopamine-immunoreactive (DA-IR) neurons in the adult insect brain has not yet been worked out in detail. Budnik and White[6] have described the distribution of DA-IR neurons in larval *Drosophila* and used glyoxylic acid for detection of catecholaminergic neurons in adults. Ca. 220 catecholamine containing (CAc) neurons were found in the CNS of Drosophila (excluding the optic lobes where hundreds of small CAc neurons were found). The CAc neurons are localized in clusters, they are few in number but invade most neuropil areas of the brain. Only interneurons were found to be CAc. Each neuron may also be invading large volumes of neuropil. Some preliminary data on DA-IR in*Calliphora* are presented in Fig. 1. In this species ca. 24 larger and 80 smaller DA-IR neurons were found in each side of the brain. Additionally, in the rind of each medulla there are hundreds of small DA-IR neurons. Little is known about the action of DA or other catecholamines in the insect CNS. Its role in sensitization has been indicated[26,45].

Fig. 2. Immunoreactive neurons in the *Drosophila* brain. The two upper tracings show schematically all the cell bodies (FMRF-IR cell bodies of the medulla omitted) in the brain in a 2-dimensional manner. The bottom panel shows a detailed tracing of FMRF-IR neurons in a 100 μm thick slab of the brain. Note long axonal projections between neuropils. The connections between the optic lobe neuropils are not shown in this tracing. Abbreviations as in Fig. 1. (Original figures.)


100µm
Serotonin
medulla
lobula
oe
sog
FMRFamide
central body
FMRFamide
calyx
Drosophila

Proctolin. Proctolin is a myoactive pentapeptide originally isolated from the cockroach[3,4]. Proctolin-immunoreactive (PL-IR) neurons are relatively scarce in the brain of studied insects[1,34]. In *Calliphora* 80-90 neurons were found in the brain and another 200-250 are present in the optic lobes[34] (Fig. 1). In contrast to the 5-HT-IR and DA-IR neurons most PL-IR neurons have processes restricted to small and restricted volumes of neuropil. For instance one set of 30 PL-IR neurons invades two layers of the central body only and another set of six neurons invades tritocerebral neuropil. Only one pair of PL-IR neurons (in protocerebrum) is known to invade extensive regions of neuropil. It should be noted that antibodies to proctolin not always label all the processes of the neurons and hence e. g. dendritic areas may not be known yet. PL-IR neurons are mainly interneurons in the brain but some are efferents (possibly antennal or mouth part motoneurons). The central action of proctolin is not yet known in insects, but a transmitter/modulator function has been suggested[39,49]. In the lobster stomatogastric ganglion proctolin initiates or increases the frequency of cycling of rhythmic motoneurons[24].

CCK- and FMRFamide-like peptides. With antisera to the molluscan cardioexcitatory peptide FMRFamide[41] distinct sets of neurons can be labeled in the insect brain[34,47,48,50]. The authentic substance(s) present in these immunolabeled neurons is not determined. In fact the FMRFamide-immunoreactive (FMRF-IR) neurons may represent a heterogenous population of neurons containing different substances cross reacting with anti-FMRFamide.

TABLE 1. Structure of some neuropeptides [1]

Proctolin [2]	Arg-Tyr-Leu-Pro-Thr-OH
FMRFamide	Phe-Met-Arg-Phe-NH_2
Met-enkephalin	Tyr-Gly-Gly-Phe-Met
YGGFMRF	Tyr-Gly-Gly-Phe-Met-Arg-Phe
Leucomyosuppressin[2]	pGlu-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH2
Leucosulfakinin I[2]	Glu-Gln-Phe-Glu-Asp-Tyr-Gly-His-Met-Arg-Phe-NH_2
Leucosulfakinin II[2]	pGlu-Ser-Asp-Asp-Tyr-Gly-His-Met-Arg-Phe-NH_2
Cholecystokinin (CCK_8)	...Met-Gly-Trp-Met-Asp-Phe-NH_2
Bovine pancreatic polypeptide	...Leu-Thr-Arg-Pro-Arg-Tyr-NH_2
Small cardioactive pept. (SCP_B)	Met-Asp-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2

[1] References to original work are given in the text. [2] Isolated from insect nervous tissue.

So far a few substances have been isolated from the insect head that may cross react with FMRFamide antisera: e. g. a pancreatic polypeptide like substance[15], leucosulfakinin I and II[28] and leucomyosuppressin[20] (see also Table 1 for peptides with sequences similar to FMRFamide). Antisera to CCK, pancreatic polypeptide and YGGFMRF label different portions of the FMRF-IR neurons in the brain of *Calliphora* and *Drosophila*[38]. In *Drosophila* ca. 150 FMRF-IR neurons could be counted in the brain and a few hundred in the optic lobe (Fig. 2). Only ca. 28 of these were recognized in wholemount preparations[50] showing that this method is not sufficient for accurate description of neuron distribution and morphology. In contrast to the proctolin-immunoreactive neurons the FMRF-IR neurons invade most brain neuropils. Some neurons are large with extensive arborizations,others are of limited extension. Several FMRF-IR pathways can be seen interconnecting different centers, but also local circuits are present (Fig. 2). Since we do not know whether the FMRF-IR neurons represent a heterogenous population with several different peptides or a single population, the complexity of the system cannot yet be appreciated. It seems that whatever the substance(s) is (are) numerous circuits and pathways are involved at many levels of integration. Only peripheral functions of FMRFamide-like substances in insects have been analyzed[8,16]. It should also be noted that we may be dealing with co-occurrence of different peptides in some portions of the FMRF-IR population. In fact, co-localization of peptides has been indicated in the brain and thoracic ganglia of *Calliphora*[13,14]: antisera to Met-enkephalin, YGGFMRF, FMRFamide, pancreatic polypeptide and gastrin/CCK specifically stain certain neurons.

NEUROACTIVE SUBSTANCES OF THE VISUAL SYSTEM

The visual system of insects, especially flies, has been investigated physiologically at the level of phototransduction and photoreceptor/first order synapses as well as at the level of identified motion sensitive units[19,42]. The motion detection and visual orientation has also been analyzed behaviourally[19]. Since the neuroanatomy is well known in flies at both levels and the systems are amenable to experimentation, pharmacological/physiological analysis has been initiated to study the role of chemical messengers in visual processing[7,17,18]. Much is yet to be learned and one step is to characterize the neuroactive substances in the visual system and the circuits they serve.

Before entering a discussion of the interneurons of the optic lobes a few comments will be made on the possible transmitters of the photoreceptors. Available pharmacological and physiological information to date suggests histamine as the transmitter in the six large (short axoned) receptors R1-R6 of the blowfly[17,18]. One of the long receptors (R7) has been reported to be GABA-immunoreactive[11], but no further evidence for a transmitter function of GABA in photoreceptors is available.Similarly the amino acid taurine is present in the retina, but pharmacological experiments failed to show a taurine mediated action on second order neurons mimicking that of the photoreceptors[17,18,51].

GABA and acetylcholine. The optic lobes of investigated insect species contain large numbers of GABA-IR neurons: on each side there are ca. 9000 in *Apis*, 10000 in *Calliphora* and 18000 in *Manduca* [21,27,43]. The high numbers are due to many of the neurons being small field units present in each retinotopic column. In *Calliphora* ca. 4000 centrifugal columnar elements (C2) connect the medulla to the lamina[11,27]. The action of these probably inhibitory feedback circuits is well suited for analysis[18] since the outputs in the lamina are identified[42]. In the medulla layers of GABA-IR neurons can be seen. These are derived from columnar neurons connecting to the lobula complex, others are large field neurons from the midbrain. In the lobula plate of *Calliphora* and *Musca* there are large ipsi- and bilateral GABA-IR neurons that correspond to identified neurons in the motion detection pathway. Also these seem useful for functional analysis. The effect of picrotoxin (blocks GABA-activated inhibitory chloride channels) on motion detection in *Calliphora* was studied and it was found that directional sensitivity of one identified neuron as well as the optomotor turning reaction were abolished[7].

In the optic lobes acetylcholine (Ach) is synthesized and stored, α-bungarotoxin binding can be demonstrated and antisera to choline acetyltransferase label masses of neurons[5,18,25]. Cholinergic neurons seem to include columnars, tangentials and amacrines. The different cholinergic circuits are, however,so far not unravelled anatomically.

Serotonin. The 5-HT-IR neurons are described in some detail in many insect species[29,30,33,35,37]. In *Calliphora* and *Drosophila* there are two major types of 5-HT-IR neurons invading optic lobe neuropils (Fig. 3A): 1) a pair of large neurons each bilaterally invading all optic lobe neuropils and caudal protocerebral neuropil and 2) wide field amacrine neurons invading certain layers of the medulla (and in *Calliphora* the lobula). There are ca. 20 of these in each lobe of *Calliphora* and 10 in *Drosophila*. The large bilateral 5-HT-IR neurons may be involved in modulating synaptic transmission over the entire visual field in a synchronized bilateral manner (affecting several integrational levels). Being the only 5-HT-IR neurons with branches in the peripheral part of the visual system they constitute a system that could be probed for 5-HT action in peripheral visual processing. Preliminary pharmacological studies in the peripheral visual pathway[12] indicate that applied 5-HT has long term effects on the sensitivity of the fly eye.

Dopamine. Little is known about dopaminergic neurons in the insect optic lobe. Using histofluorescence techniques it has been demonstrated that the medulla and lobula of most insects contain catecholaminergic neurons[6,22]. These seem to be derived from cell bodies in the midbrain (in *Drosophila* numerous additional small cell bodies can be seen distal to the medulla[6]). With improved immunocytochemical techniques hopefully these neurons may be labeled in more detail.

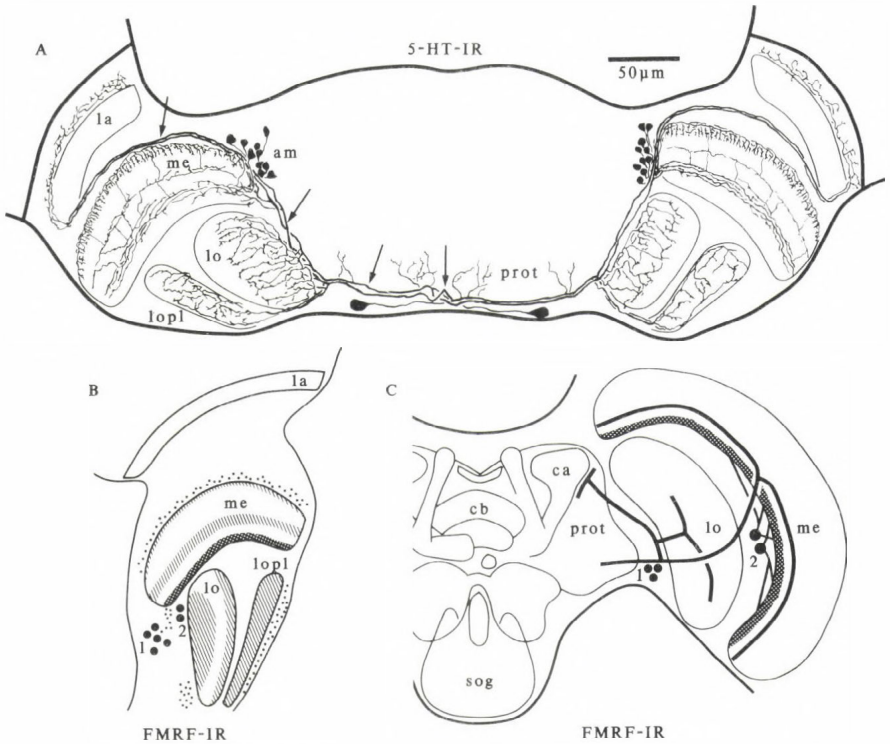


Fig. 3. Tracings of optic lobe neurons. A. 5-HT-IR neurons in the *Drosophila* optic lobes. Two large bilateral neurons (arrows on one side) invade all optic neuropils. A set of amacrine (am) neurons invade the medulla only. B. Schematic horizontal view of FMRF-IR neurons in *Calliphora* optic lobe. Filled circles depict cell bodies and stippling layers of processes. The inner medulla layer is derived from the two amacrines (2). C. Schematic diagram of two of the FMRF-IR neuron types. One set (1) invades medulla, lobula and lateral horn of protocerebrum (prot). The other (2) consists of two large amacrines. Altered from Refs. 29,38. These neurons are potentially identifiable.

Proctolin. Neurons in the lobula of cockroaches and flies react with antibodies to proctolin[34]. In *Calliphora* there are ca. 100 PL-IR neurons in each lobula. Their small cell bodies are located ventrally at the base of the lobula and they have fine varicose processes in a broad proximal layer of the lobula neuropil. Although their axonal processes are not stained in their entire length it is likely that these neurons connect to midbrain centers. The very restricted distribution of PL-IR is intriguing since most other immunoreactive neurons can be found also in the medulla and the lobula plate.

FMRFamide-like peptides. With antisera to FMRFamide, CCK, pancreatic polypeptide and YGGFMRF, immunoreactive neurons have been found in different patterns in the optic lobes of *Calliphora*[38]. No immunoreactive neurons are present in the lamina. All antisera stained neurons in the medulla, but in the lobula and the lobula plate some differences in staining could be seen. The medulla is supplied with at least four types of FMRF-IR neurons (Figs. 3B,C). Two very large amacrine FMRF-IR neurons were found to invade the entire retinotopic mosaic of the medulla in a proximal thin layer. Two types of tangential neurons (totally less than 10 neurons) connect the medulla to the midbrain and there are hundreds of FMRF-IR neurons with cell bodies distal to the medulla. The lobula is supplied by one of the tangential neuron types that also supply the medulla and the midbrain and the lobula plate is innervated by numerous small amacrine FMRF-IR neurons. The FMRF-IR neuron type that supplies the medulla and the lobula sends their axons to a neuropil adjacent to the calyx of the mushroom body (Fig. 3C). In this neuropil (the lateral horn) they arborize among terminals from the chemosensory system and the ocellar system. This pathway seems to be multimodal and merits functional analysis. CCK-IR can be seen in the tangential neurons connecting medulla, lobula and lateral horn, in the numerous small medulla neurons and in the lobula plate neurons. All CCK-IR neurons are included in the FMRF-IR population. Antisera to pancreatic polypeptide stain all FMRF-IR neurons except medulla-lobula-lateral horn neurons. The YGGFMRF antiserum reacts only with the large medulla amacrines. These findings indicate that we probably are dealing with a heterogenous population of FMRF-IR neurons in the optic lobes. The total population labels with the "least specific" antiserum (anti-FMRFamide) and other more "narrow" antisera recognize subpopulations. Further immunological and biochemical studies are needed to characterize the FMRF-like circuits in the optic lobes before meaningful pharmacological experiments can be performed.

CONCLUSIONS

Immunocytochemistry has provided maps of distributions and morphology of insect brain neurons defined by their putative neuroactive substances. Although only relatively few substances have been investigated a complex picture has emerged. In most cases the immunocytochemically identified circuits are so complex and widely distributed in the brain that each small circuit has to be probed physiologically/pharmacologically for its function.

Appropriate centers, circuits or pathways can be selected for such analysis and local actions matched with overall alterations in different behaviors induced by pharmacological or genetical manipulations of the transmitter systems. The optic lobes of insects seem to offer a good model for such studies. The neurons reacting with antisera tested so far only account for a very small portion of the brain and optic lobe neurons. In some centers, different neurons

seem to react with most of these antisera, e. g. in the central body[30,31,34] in others, like e. g. the mushroom bodies and the protocerebral bridge, few immunoreactive neurons have been resolved. Future work probably will reveal further neuroactive substances in the insect brain. Never will the nervous system be what we thought it was: a network of linear connections where signals are transmitted by a few chemical messengers. The neural occurrence and co-occurrence of a large number of substances, most of whose actions are not known in the CNS, indicate that synaptic transmission is extremely complex and that we have to think of combined actions of substances at the synapses. In insects the neuropharmacology of the CNS is in its infancy and efforts have to be made at studying receptors, synaptic connections of chemically identified neurons and pharmacological actions on signal processing in identified systems.

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DISCUSSION

WALKER, R.J.: Did you find any evidence for the co-localization of peptides and monoamines?

NÄSSEL, D.R.: In our studies of Calliphora and Drosophila no clear demonstration of co-localization of peptides and monoamines has been made. As mentioned in my paper, several cases of peptide co-localization appear in the brain and thoracic ganglia. There are, however, some clusters of small peptidergic neurons which may also be dopamine-immunoreactive. This will be tested by protein A-gold immunolabelling of adjacent sections with the different antisera.

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NEUROSECRETORY CELLS OF THE LOCUST SUBOESOPHAGEAL GANGLION

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INTRODUCTION

The suboesophageal ganglion of locusts has eight pairs of lateral nerves (see Fig. 2) and an unpaired posterior median nerve which innervate the mouthparts and the neck (Altman and Kien, 1979). In addition to these major peripheral nerves an interconnected system of small diameter nerves has recently been discovered (Bräunig, 1987): These small nerves follow the big lateral nerves (except nerves 3 and 8) into distal regions, branch where the big ones branch, and have for that reason been named 'satellite nerves'. The entire system of satellite nerves is established by only three neurons on each side of the suboesophageal ganglion. Their somata range between 35 and 40 µm in diameter and are located near the anterior margin of the ganglion, between the roots of the circumoesophageal connectives. Within the ganglion their central projections cover the dorsal margin of the ipsilateral mandibular neuromere and also form a vertical, parasagittal band of terminals on the border to the maxillar neuromere (see Fig. 1D).

Axons of the satellite neurons exit from the ganglion via the mandibular nerve (nerve 1), which subdivides into three major branches (see Fig. 2, nerves 1B, 1C, 1D; nerve 1A is a minor branch innervating the hypopharynx). Usually all satellite nerves originate from branch 1C as a common bundle (see Fig. 2, ST), but there is much variation in the branching pattern.

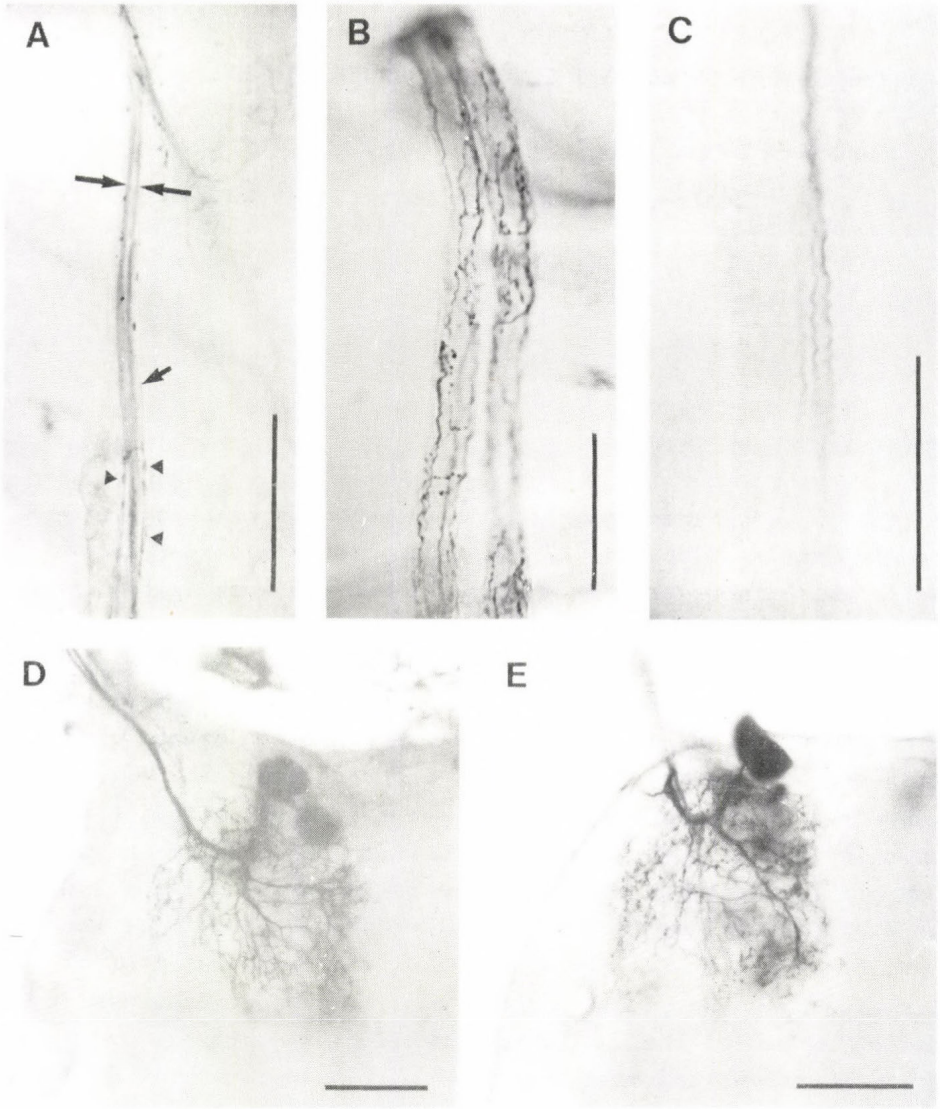

Fig. 1. Morphological features of serotonin-immunoreactive neurosecretory cells of the locust suboesophageal ganglion.

A: A satellite nerve with two core axons (long arrows). From one axon emerges a side branch (short arrow) that terminates in bleb-like endings (arrowheads) on the surface of the nerve (anti-serotonin staining). Scale: 50 µm. B: Satellite neuron meshwork on the surface of nerves 6 and 7 of the suboesophageal ganglion (anti-serotonin staining). Scale: 100 m. B: Satellite neuron meshwork on the surface of nerves 6 and 7 of the sub-oesophageal ganglion (anti-serotonin staining). Scale: 100 m.

In the periphery the satellite nerves establish a dense meshwork of varicose terminals on the surface of the peripheral nerves of the suboesophageal ganglion and the tritocerebral nerves which innervate the labrum. Thus only six suboesophageal neurons establish a system of axon collaterals and nerve terminals which almost completely covers the outer surface of all major peripheral nerves in the ventral half of the head.

Similar fine meshworks of varicose nerve terminals on the outer surface of the nerves innervating the mouthparts had already been revealed in flies (Nässel and Elekes, 1985) and cockroaches (Davis, 1985) by means of serotonin-immunocytochemistry. Also, serotonin-immunoreactive cell bodies located anteriorly within the suboesophageal ganglion with axons in the mandibular nerve had been previously observed in locusts (Tyrer et al., 1984) and other insects (Bishop and O'Shea, 1983; Flanagan, 1986).

These results suggested that the satellite nervous system of locusts and the so-called 'serotonin neurohemal system' (Davis, 1985) of other insects might be comparable structures revealed by different methods. It seemed worthwhile, therefore, to find out whether elements of the locust satellite nervous system could be stained with antibodies against serotonin.

METHODS

The immunocytochemical procedures used were essentially those of Tyrer et al. (1984). Details concerning cobalt histology and electrophysiological methods are given by Bräunig (1987).

←

C: Link nerve axons in the motor nerve of the mandible opener motor nerve 1C. Three axons are clearly visible, the fourth is outside the focal plane (anti-serotonin staining). Scale: 100 µm. D, E: Satellite neurons (D) and link nerve neurons (E) in comparison. Not all cell bodies are in the focal plane (silver-intensified cobalt backfills of nerves ST (D) and LN (E)). Scales: 100 µm

RESULTS

Immunocytochemical aspects of the satellite nerves

As can be seen in Fig. 1, the satellite nervous system is indeed serotonin-immunoreactive. Both the satellite nerves and their terminals are stained with this method. Not previously revealed by cobalt staining was that the satellite nerves themselves bear terminals on their surfaces: Short collaterals leave the axons which run in the core of a satellite nerve and terminate in bleb-like structures on their surface (Fig. 1A).

The surface meshwork is particularly dense on nerves 1A, 2, 6 and 7 of the suboesophageal ganglion (Fig. 1B) and on the tritocerebral nerves innervating labral muscles and sense organs. On the surface of mandibular nerves the meshwork is sparse and does not extend much beyond the link nerve junctions (see below). This result is supported by electrophysiological studies. Furthermore immunocytochemistry supports previous results (Bräunig, 1987) that nerves 3, 8 and the median nerve do not receive satellite innervation: No immunoreactive structures could be seen on these nerves.

Other serotonin-immunoreactive neurons

Immunocytochemistry revealed a second group of axons that do not leave nerve 1C to enter the satellite nerves. This second group could be traced within nerve 1C far out into the periphery (Fig. 1C). Shortly before nerve 1C ramifies to innervate the mandibular opener muscle (M8) the serotonin-immunoreactive axons enter a nerve (LN in Fig. 2) that contains a few sensory fibres and links the opener nerve 1C to nerve 1D, the motor nerve of the closer muscle (M9).

Immunocytochemistry as well as cobalt backfills of the link nerve (LN) show a maximum of four axons or cell bodies in this second group of cells. Within this group there are two types with two neurons each: Type I cells reach the closer muscle via the opener nerve (1C) and the link nerve (LN) (Fig. 2, dotted lines). Type II cells in addition take a direct route towards the closer muscle via nerve 1D (Fig. 2, dashed lines).

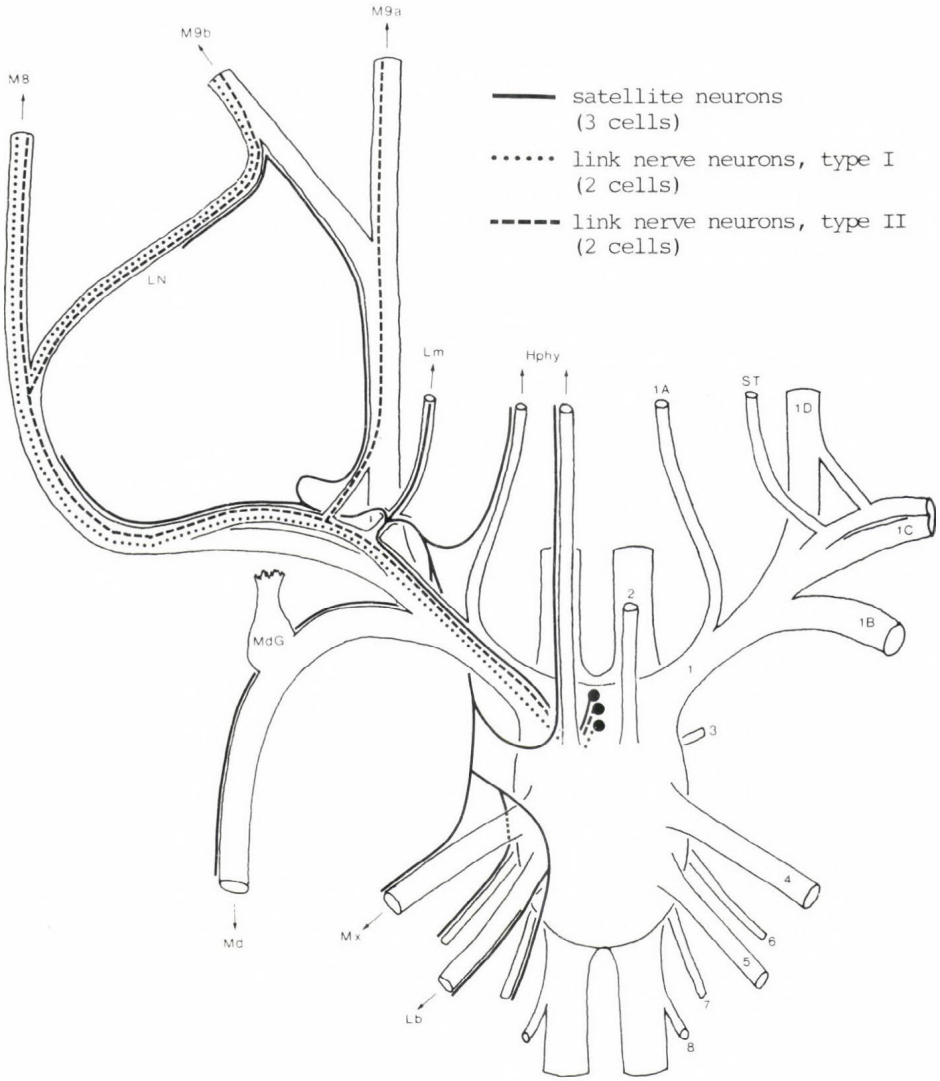

Fig. 2. Schematic representation of the three types of sub-oesophageal serotonin-immunoreactive neurons with the course of their axons in the peripheral nervous system. For each type only one cell is represented, total numbers are given in parentheses. The nerves of the ganglion are numbered on the right according to the system of Altman and Kien (1979). On the left the destinations of these nerves are indicated. Abbreviations: Hphy = hypopharynx; Md = mandible; MdG = mandibular ganglion; M8 = mandibular opener muscle; M9a, M9b = medial and lateral parts of mandibular closer muscle; Mx = maxilla; Lb = labium; Lm = labrum; LN = link nerve; ST = satellite nerve to tritocerebral (= labral) nerves)

It is not yet clear whether both types, from the junction with the link nerve, proceed further within nerve 1C towards the opener muscle (as indicated in Fig. 2), or only one of them.

The 'link nerve neurons' establish a superficial network of beaded terminals on the surface of both the opener and the closer muscle motor nerves similar to the one established by the satellite neurons on other nerves. The meshwork is limited to the nerves. Terminals on muscle fibres were in no case observed. Thus, these neurons appear to be a second group of satellite neurons responsible to cover peripheral nerve surfaces in the dorsal half of the head, particularly the numerous branches of nerve 1D within the powerful closer muscle M9.

Common features of the serotonin-immunoreactive neurons

As stated above cobalt backfills of the link nerve reveal a maximum of four cells within the suboesophageal ganglion. By their central arborizations these cells are indistinguishable from the satellite neurons (Fig. 1 D,E), but the course of their axons in the periphery and electrophysiological experiments (Bräunig, unpublished) clearly set them apart from those.

In about 10% of all specimens studied the axons of both groups of serotonin-immunoreactive cells form a common nerve that separates from the main nerve 1C for a short distance (as indicated in Fig. 2). From here three of the seven axons enter the various branches of the satellite nervous system, four proceed further distally towards the link nerve. The number of seven axons on each side corresponds well with the maximum number of 14 immunoreactive cell bodies anteriorly within the suboesophageal ganglion that can be observed in sectioned material (Tyrer, personal communication).

DISCUSSION

The serotonin-immunoreactive system of the suboesophageal ganglion of various insect species

In the locust, as in other insects, there is a serotonin-immunoreactive network on the surface of the peripheral nerves of the mouthparts. Generally, the serotonin-positive systems are comparable in the three species so far studied: fly (Nässel and Elekes, 1985), cockroach (Davis, 1987) and locust. They differ very much, however, where details are considered.

In the cockroach, for example, the axons of the suboesophageal neurons reach the labral nerves after taking a circuitous route via the circumoesophageal connectives (Davis, 1987). In the locust there is a much more direct pathway via the two ST nerves (ST = satellite nerve to tritocerebrum) which circumnavigate the pharynx laterally (Bräunig, 1987).

As shown here, in the locust the serotonin-positive network is established by 14 neurons of the suboesophageal ganglion. This number appears exceptionally large when compared to other insects. Recently it has been shown that the corresponding 'serotonin neurohemal system' of cockroaches originates from only 4 to 6 neurons in the suboesophageal ganglion (Davis, 1987). Similarly, the corresponding system in flies appears to originate from 4 large serotonin-positive cells (Nässel and Elekes, 1985). Also in dragonflies (Longley and Longley, 1986) and hemipteran insects (Flanagan, 1986) only 4 serotonin-immunoreactive cell bodies of comparable size and location have been found within the suboesophageal ganglion.

Apart from differences between species this discrepancy in numbers might also be explained on methodological grounds. Some results suggest that the method reveals the complete set of cells only in exceptional cases. For instance in the wholemount preparations used here, 7 axons (3 satellite and 4 link nerve axons) in each mandibular nerve can only be seen in particularly well stained preparations. On average there are 2 satellite axons and 2 link nerve axons clearly visible. Correspondingly, 14 serotonin-positive cell bodies in sectioned material is the

maximum number observed. Usually only 6 to 8 somata can be seen (Tyrer et al., 1984). Probably individual cells at certain times do not contain enough antigen and therefore the method usually reveals only subpopulations of cells.

In contrast, the other methods applied (cobalt backfills and electrophysiology; Bräunig, 1987) do not hint at variations of cell numbers between specimens. There might be individual locusts with only 3 link nerve neurons, but the number of 3 satellite neurons on each side of the suboesophageal ganglion was found to be absolutely constant.

Innervation of motor nerves

Serotonin-positive nerve terminals were only found on the outer surface of peripheral nerves. The meshwork does extend into muscles and it does cover the surface of individual motor nerve branches within a muscle, but it was never observed on the surface of muscle fibres.

The following observation makes it unlikely that this results from a failure of the method: On certain muscles in the labrum serotonin-immunoreactive terminals could be found. These, however, appeared to be discontinuous with the satellite neuron ramifications. They probably stem from another source, most likely the frontal ganglion. This shows that the method is able to detect such terminals on muscle fibres, when they are present.

Functional aspects

Although there is as yet no direct evidence that the serotonin-immunoreactive cells of the locust suboesophageal ganglion are neurosecretory, for several reasons previously discussed (Bräunig, 1987) this interpretation is the most likely one at present. The general interpretation of the results obtained in various insect species is that the system serves a neurohemal function for the release of serotonin (Nässel and Elekes, 1985; Davis, 1985, 1987; Bräunig, 1987).

For reasons unknown the serotonin-immunoreactive cells do not establish distinct neurohemal organs like, for instance,

the corpora cardiaca or the median nerve perivisceral organs. They rather spread their release sites over the surfaces of nerves and ganglia. In this they resemble peripheral neurosecretory cells (Finlayson and Osborne, 1968). An interesting hypothesis has been proposed by Davis (1987): The serotonin neurohemal system might be ideally suited to set local pulses of neurosecretion during feeding. The arrangement of the release sites could be a means to influence the mouthpart target organs and at the same time to avoid loading the entire hemolymph volume with serotonin.

ACKNOWLEDGEMENTS

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DISCUSSION

KISS, T.: In the stick insect, along the link nerve there are four or five neurons. Is there any analogy between these neurons and those which were discussed by you?

BRÄUNIG, P.: First, the "link nerve" I describe here is in no way homologous to the stick insect link nerve. There is, however, an analogy between peripheral neurosecretory cells in stick insects and the locust 5HT-neurons: both use the outer surface of peripheral nerves as a substrate for their efferent terminals.

NÄSSEL, D.R.: Did you find any serotonin-immunoreactive terminals in the neural sheath of thoracic or abdominal nerve roots?

BRÄUNIG, P.: Only the prothoracic and unfused abdominal ganglia have been investigated immunocytochemically. There are no serotonin-immunoreactive profiles. There are, however, small nerves originating from abdominal ganglia which resemble the satellite nerves of the suboesophageal ganglion.

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5,6-DIHYDROXYTRYPTAMINE INDUCED ULTRASTRUCTURAL CHANGES AS A SPECIFIC MARKER OF THE SEROTONERGIC SYSTEM IN THE CNS OF HELIX POMATIA

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INTRODUCTION

As described earlier, the false-transmitter 5,6-dihydroxytryptamine (5,6-DHT), injected into the body cavity of Helix pomatia, induces brown pigmentation in special groups of neurons of the central nervous system (CNS), without impairing their electrophysiological characteristics following formation (S.-Rózsa et al., 1986; Hernádi et al., 1987). The application of 5,7-DHT also induces pigmentation similarly to 5,6-DHT in identified serotonergic neurons of different invertebrate species, as in Hirudo medicinalis (Glover and Kramer, 1982; Lent and Dickinson, 1984), Helix lucorum (Balaban et al., 1985) and Aplysia californica (Jahan-Parwar et al., 1986). The pigment-induced neurons are supposed to be serotonergic since they include all the serotonergic neurons that have already been identified (Balaban et al., 1985; S.-Rózsa et al., 1986; Jahan-Parwar et al., 1986). In gastropods, in the course of treatment with 5,6-DHT for 8-14 days, the electrophysiological, biochemical and behavioural tests demonstrated a decrease in the synaptic efficacy of the serotonergic synapses of the metacerebral giant neuron (MGC), in respect to the serotonin level and feeding arousal (see: Vehovszky et al., Kemenes et al., in this Volume).

This communication aims at supporting the hypothesis that the pigment-induced neurons are serotonergic, using 5-HT immunocytochemistry in whole mount preparations. In addition, the observable ultrastructural alterations in the CNS are fol-

lowed throughout the period of treatment, and are compared to the changes demonstrated by electrophysiological, biochemical and behavioural tests.

RESULTS

Comparison of pigment-induced neurons with 5-HT immunocytochemically labelled neurons

The 5-HT immunopositive neurons can be found in the same ganglia as those neurons detected following the pigment-induction technique (S.Rózsa et al., 1986; Hernádi et al., 1987). For detaliled description of the 5-HT positive elements see the papers by Elekes et al. (in this Volume) and Hernádi et al. (in preparation). According to the detailed topographic analysis, the number and location of the 5-HT immunopositive neurons show close correlation with that of the pigment-induced neurons (Fig. 1). This is supportive of our earlier hypothesis that all the pigment-induced neurons exhibit serotonergic characteristics (S.-Rózsa et al., 1986; Hernádi et al., 1987).

Time-dependent ultrastructural alterations of the CNS in the course of 5,6-DHT treatment

In the first period of the treatment the most striking alteration is the extreme dilatation of the extracellular spaces, especially in the fine neuropil areas of the CNS (Fig. 2). Parallel with this, dense deposits are detectable on the membrane surfaces of different kinds of fibres 30 min after the injection (Fig. 2). After 2-3 days of treatment certain types of the neuronal fibres show increased density, having empty and dense-core vesicles (Fig. 2). The ultrastructural changes described can be observed mainly in the first week of the treatment. Later lamellar membranous structures are visible in a special type of fibre and synaptic structures, increasing in frequency in the course of time (Fig. 3). This type of fibre and synaptic structures usually possesses glycogen particles, as well as dense core and clear vesicles (Figs 3 and 4). There

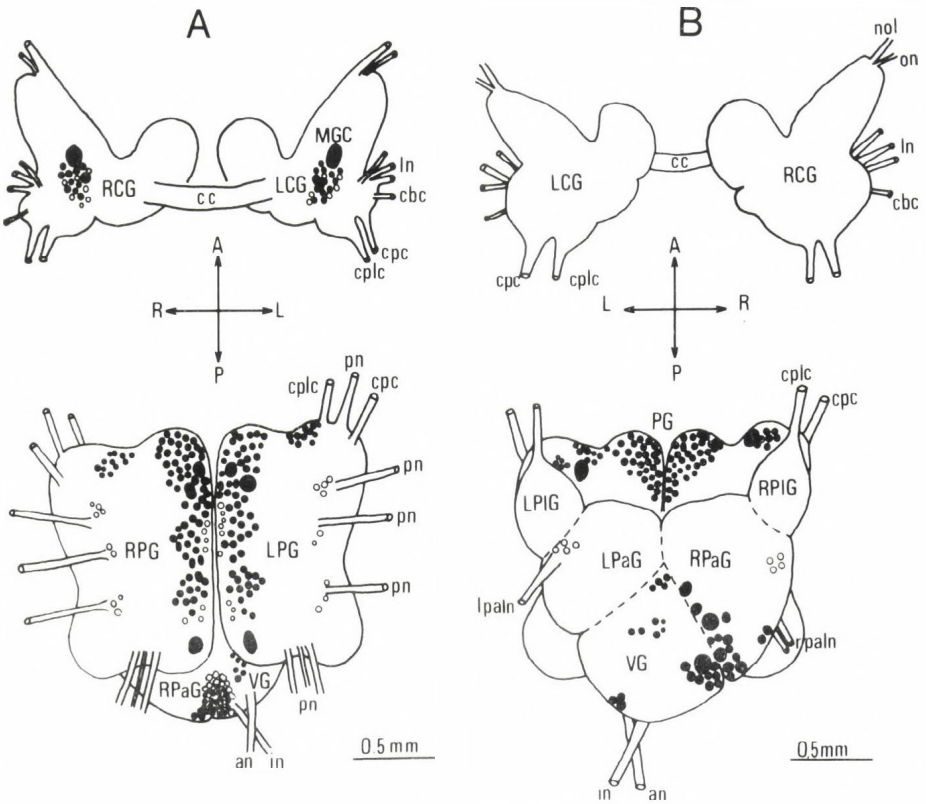


Fig. 1. Schematic representation of the topographic distribution of 5-HT immunoreactive and 5,6-DHT-induced pigmented neurons in different ganglia of the CNS. Black circles: neurons showing both 5-HT immunoreactivity and 5,6-DHT-induced pigmentation. Open circles: neurons showing only 5-HT immunoreactivity. A: ventral surface, B: dorsal surface. A - anterior, P - posterior, L - left, R - right, RCG - right cerebral ganglion, LCG - left cerebral ganglion, PG - pedal ganglion, RPG - right pedal ganglion, LPG - left pedal ganglion, RPlG - right pleural ganglion, LPlG - left pleural ganglion, RPaG - right parietal ganglion, LPaG - left parietal ganglion, VG - visceral ganglion, cc - cerebral commissure, nol - olfactory nerve, on - optic nerve, ln - labial nerve, cbc - cerebro-buccal connective, cpc - cerebro-pedal connective, cplc - cerebro-pleural connective, pn - pedal nerve, rpaln - right pallial nerve, lpaln - left pallial nerve, in - intestinal nerve, an - anal nerve, MGC - metacerebral giant cell.

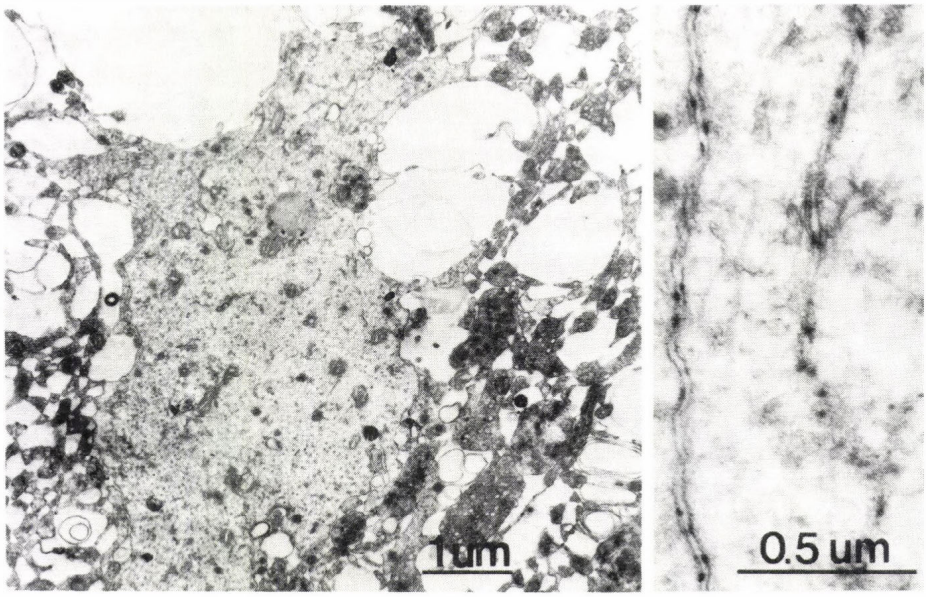


Fig. 2. After 2 days of treatment 5,6-DHT evokes dilatation of the extracellular spaces as well as an increase in the density of the terminals in the fine neuropil areas of the CNS. Insert: Small dense deposits can be seen on the neuronal membrane surfaces in the first phase of the treatment.

is an increase in the number of rolled up membranous structures, reaching the highest frequency between the 14th and 20th day of treatment. By this time, numerous membranous structures are also detectable in the cytoplasm of the neuronal somata. These rolled up membranous structures are present in the fibres and synaptic structures even after 60 days of treatment, although at a lower frequency. At this point the density of the glycogen particles increases in the fibres and synaptic structures (Fig. 5). In the meantime the ultrastructure of the CNS returns to normal following 20 days of 5,6-DHT treatment.

In order to elucidate whether the rolled up membranous structures are specific to the serotonergic neurons, the MGC was injected with horse radish peroxidase (HRP) 2 weeks after the 5,6-DHT injection, at the time when the membranous structures showed the highest density. It was found that in the

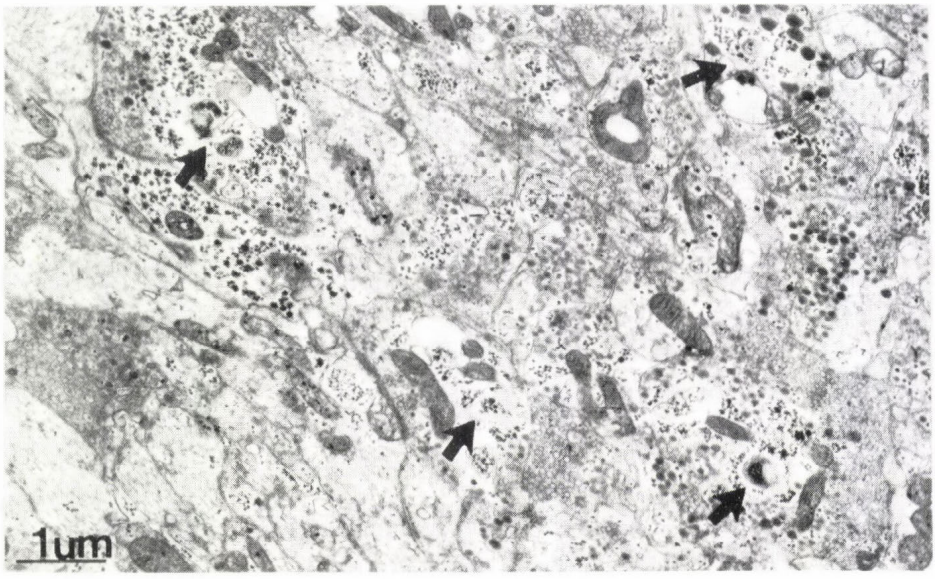


Fig. 3

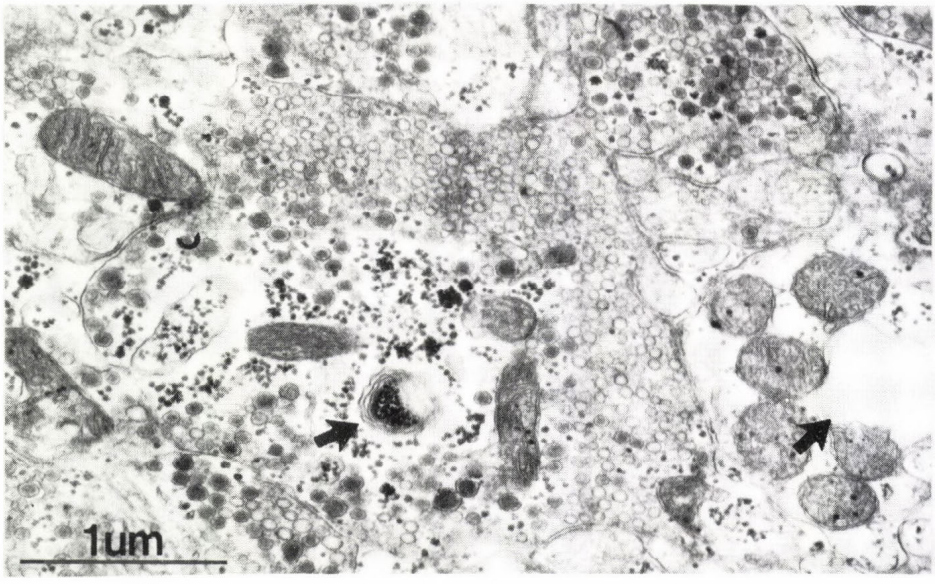


Fig. 4

Figs 3 and 4. Photographs taken 14 days after 5,6-DHT treatment demonstrate rolled up membranous structures (arrows) in numerous fibres and synaptic structures having many glycogen particles (Fig. 3). The high magnification photograph of a synaptic structure shows a rolled up membranous structure enclosing glycogen particles (arrow) (Fig. 4).

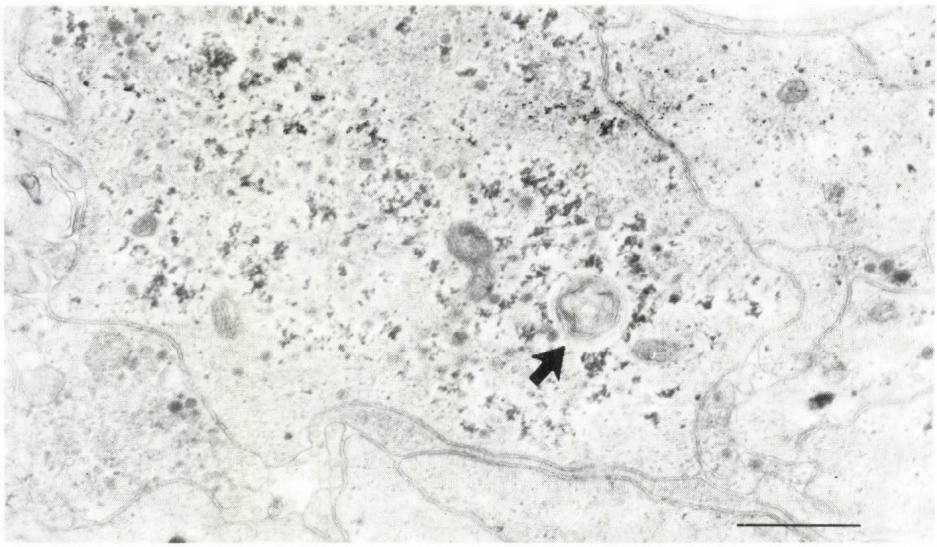
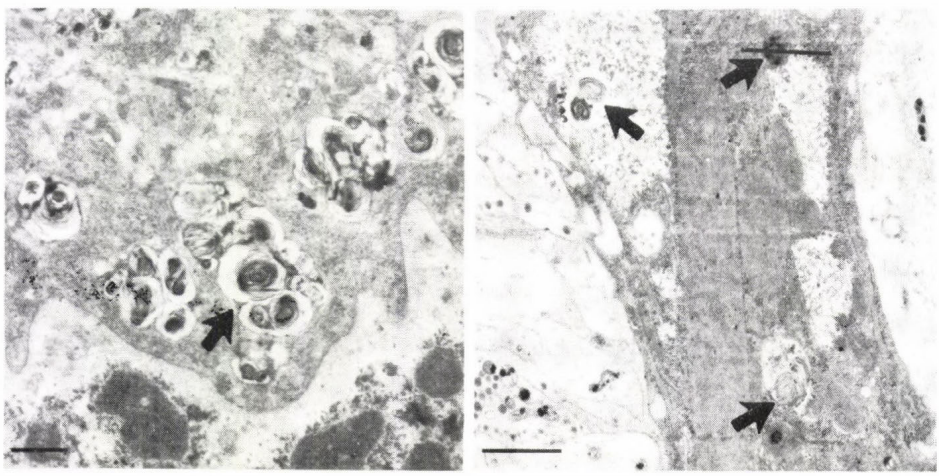


Fig. 5. After 60 days of 5,6-DHT treatment the rolled up membranous structures are still detectable (arrow) in the synaptic profiles and fibres, although in lower number. The merged glycogen particles show high density appearances.



(A) (B)

Fig. 6. After 14 days of 5,6-DHT treatment numerous membranous structures are observable (arrows) in the HRP-labelled serotonergic MGC both in the cytoplasm (A) and in the processes (B).

HRP-labelled cytoplasm, as well as in the labelled fibres of the MGC, numerous membranous structures are present (Fig. 6).

DISCUSSION

Based on the results obtained it can be stated that in the first phase of the treatment the false transmitter 5,6-DHT has a nonspecific general effect on the ultrastructure of the neuronal fibres and synaptic structures. Later, however, from the 8th to 10th day, the effect becomes specific, inducing rolled up membranous structures in the serotonergic fibres, synaptic elements and somata, which can also be clearly demonstrated in the HRP labelled metacerebral giant cell (MGC). The appearance and increase in the number of the rolled up membranous structures in the neuronal elements between the 8th and 14th day coincide with the changes in the efficacy of the synaptic connections of the serotonergic MGC, as well as in the serotonin level of the CNS (Vehovszky et al., in this Volume).

After 20 days of treatment, when pigmentation of the serotonergic somata has developed, the general ultrastructure of the central nervous system tends to show normal appearance again, by which time the electrophysiological, biochemical and behavioural tests demonstrate functional recovery (Vehovszky et al., in this Volume).

According to the close correlation between 5-HT immunocytochemistry and 5,6-DHT-induced pigmentation, the developing pigmentation can be considered specific of the serotonergic somata. The 5,6-DHT-induced rolled up membranous structures in the synaptic structures, seen in the fibres as well as in the somata during the restoration phase, may be considered as specific markers of the serotonergic neurons, which also enables the use of this <u>in vivo</u> labelling technique at ultrastructural levels.

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DISCUSSION

MOFFETT, S.: How does the ultrastructure of cells stained with 5,7-DHT differ from that obtained with 5,6-DHT?

HERNÁDI, L.: The number and location of the pigmented neurons evoked by 5,7-DHT were the same as those after 5,6-DHT injection, but the ultrastructural changes have not been investigated in Helix.

PENZLIN, H.: What about behavioural changes of the snail after application of 5,6-dihydroxytryptamine?

HERNÁDI, L.: After injection of the neurotoxin general behavioural changes can be observed as an activity increase. The fine behavioural changes concerning the feeding behaviour during the time course of the treatment will be demonstrated in a separate presentation (Vehovszky et al., in this Volume).

ROUBOS, E.W.: In one of your slides cells treated with 5,6-dihydroxytryptamine showed a nucleus with strongly aggregated chromatin. Would this aggregation be an easy criterion for the identification of 5-HT producing cells?

HERNÁDI, L.: The strong aggregation in the nucleus was evoked by the HRP injection but not by the 5,6-DHT treatment. This type of aggregation in the nucleus of serotonergic neurons has not been observed at any applied incubation time.

SAKHAROV, D.A.: Do you have any idea on the similarity between two sorts of darkening observed in 5-HT neurons, one induced by 5,6-DHT and another associated with a functional depression

of serotonergic mechanisms? (I mean the finding on leech reported here during the morning session by Dr. Leake.) Small amounts of 5,6-DHT are reportedly present in the nervous system. It seems probable that this endogenous 5,6-DHT could control the level of serotonergic activity, could it?

HERNÁDI, L.: The reddish brown orange autopigmentation observed in Helix neurons during the winter, involving the serotonergic neurons, probably represents serotonin storage. It was demonstrated earlier (Schwartz et al., 1979) that the 5-HT excess is stored in lysosome-like structures which are analogous with the pigment granules observed after 5,6-DHT injection. The 5-HT level can be regulated by storage in autopigment granules that evoke general activity changes, as it was demonstrated in leech by Dr. Leake. Since the colour of the autopigment is totally different from that of the 5,6-DHT induced pigments, we suppose that the form of storage of 5-HT in the autopigments is not the 5,6-DHT.

BALABAN, P.M.: Who was the first to apply the in vivo labelling technique of serotonergic neurons of gastropods?

HERNÁDI, L.: The 5,7-DHT was the first neurotoxin applied by your group to label serotonergic neurons of gastropods in vivo. This was referred to in our paper dealing with the in vivo labelling of the serotonergic neurons by 5,6-DHT in Helix CNS (S.-Rózsa, K. et al., 1986) and in our poster presented in Amsterdam with your co-authorship (Vehovszky, Á., Hernádi, L., Balaban, P.M., S.-Rózsa K. SYMON Amsterdam, 1986).

Nevertheless, this presentation is dealing not with the fact that the neurotoxin induces visible pigments in the serotonergic neuron, but with its specificity and its time-dependent ultrastructural development.

S.-RÓZSA, K.: (Comment) Presumably Dr. Hernádi did not make it sufficiently clear that we described the labelling of the serotonergic cells using neurotoxin 5,6-DHT. He does not claim to have discovered it first, as this method has been used first

on the leech by Glover and Kramer (Science, 1982, 216, 317-319) and by Lent and Dickinson (Brain Res., 1984, 300, 167-171), then 5,7-DHT was used on Helix lucorum by Balaban and co-workers (Dokl. Akad. Sci. USSR, 1985, 282, 735-738, in Russian), and finally applied to Aplysia by Jahan-Parwar and co-workers (16th Annual Meeting of American Society for Neurobiology, Washington, USA, 1986, and Brain Res., in press). We used 5,6-DHT on Helix pomatia for mapping the toxin-labelled serotonergic cells (S.-Rózsa and coworkers, Comp. Biochem. Physiol., 1986, 85C, 419-425). The results we described here firstly show the ultrastructural characterization including granule formation under the influence of 5,6-DHT and this was compared to the cell structure marked by the immunocytochemical procedure. The time course of pigment formation was also described. Now we are using this method routinely to label serotonergic cells and to combine it with other methods, including HRP, as was shown here, for more detailed characterization of the serotonergic system. A detailed study of serotonin-containing cells has become more important due to its role in the learning processes.

I would like to emphasize that we cited Balaban's work in our paper (see: S.-Rózsa, K. et al., 1986) and had a joint poster with his co-authorship last year in Amsterdam (see: Hernádi, L., Kemenes, G., S.-Rózsa, K., 1987).

SALÁNKI, J.: I would like to recall as an information to this topic that 10-12 years ago, when the false-transmitter role of 5,6-dihydroxytryptamine was described in vertebrates, we studied its effect on the mussel Anodonta cygnea. We reported besides 5-HT releasing effect also about its influence on the behaviour of the animal and on the morphological changes in the ganglia. However, that time we did not follow morphological alterations for longer than one week, and did not see any pigment accumulation. Certainly, it would be interesting to repeat these experiments with a longer (1-2 months) incubation period.

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SOME HISTOCHEMICAL ASPECTS OF INVERTEBRATE ENTERIC NERVOUS SYSTEM

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ABSTRACT

The enteric nervous system of three different invertebrate species (Lumbricus terrestris, Locusta migratoria and Helix pomatia) was studied by means of different light microscopic histochemical methods. The application of the NBT/NADH method introduced by Gabella allowed us to evaluate the location, number and size of the enteric nerve elements. We demonstrated the lack of the ganglionated arrangement in the enteric nervous system of invertebrates; only solitary cells and nerve fibres were seen. The amounts of these nerve elements were different in the different species or even within the different regions of the gut of one animal. In Lumbricus the NBT-positive cells were restricted to the pharyngo-oesophageal region of the gut, while in Helix the NBT-positive nerve elements were present in the whole length of the intestine. The foregut of Locusta was free of reactive cells, however, a large number of small NBT-positive cells were seen in the hindgut and coecum. Using glyoxylic acid induced fluorescence method we were able to demonstrate and map the catecholamine-containing nerve elements in the intestinal musculature of Lumbricus and Helix. In Lumbricus fluorescent nerve bundles and cells were seen around the pharyngo-oesophageal region of the gut. Going farther from this region the fluorescent nerve elements gradually disappeared. Nerve fibres and perikarya with an intensive green fluorescence were found in the whole length of the alimentary tract of the

snail, however, considering the distribution of the fluorescent nerve elements and also the intensity of the fluorescence, a decreasing gradient was revealed from the foregut through the stomach to the hindgut. The presence of fluorescence was season-dependent and sensitive to 6-hydroxy-dopamine. Applying immunohistochemical methods on wholemount stretch preparates of Helix intestine several GABA-positive cells were revealed. A large number of acetylcholinesterase (AChE) positive perikarya and nerve fibres were also present in the musculature of the stomach of Helix.

INTRODUCTION

Despite considerable interest in the nervous control of the digestive tract of vertebrates, the gut of invertebrates has been neglected by researchers until recently. Since the density of nerve elements in the enteric nervous system is much lower than in the central nervous system, using wholemounts to study the organization of the enteric nervous system is very useful. By now almost all the light microscopic histochemical techniques have been adapted to wholemount preparates. Gabella (1969) proposed a staining technique based on the histochemical reaction for the NADH-dependent dehydrogenase activity with nitro-blue-tetrazolium (NBT) as an electron acceptor for the demonstration of intrinsic neurons in the nervous system of the intestinal wall. It was shown that glyoxylic acid induced fluorescence is especially sensitive for the catecholamines in peripheral adrenergic nerves (Furness and Costa, 1974). Some of these techniques were also successfully adapted to wholemount preparates of gut (Fekete, 1984). Recently even the light microscopic immunohistochemical (ICC) techniques have been adapted for use in wholemounts (Costa et al. 1980, Ferri et al. 1983) and it is now even suitable for performing combined light and electron microscopic ICC studies (Llewellyn-Smith et al. 1985). As a result of all these methods it is now clear that the enteric nerves at least in higher vertebrates contain a number of different neuronal subpopulations, which can be

characterized by morphological criteria (Cook and Burnstock, 1976) or by their content of neurotransmitters or neurotransmitter candidates, which are sometimes found in coexistence with one another in single neurons (Furness and Costa, 1974, Furness et al., 1984). In the present work our main purpose was to find some phylogenetical connection in the development of the enteric nervous system between vertebrates and invertebrates. We applied some of the above-mentioned techniques on some invertebrate gastrointestinal tracts. We collected most data on the snail's alimentary canal since the intestine of Helix pomatia was very suitable for wholemount preparates. However, the alimentary canal of insects raised a lot of problems. The muscle layer in insect intestine is very thin and discontinuous and has an extreme tracheolar supply.

MATERIALS AND METHODS

Adults of different invertebrates (Lumbricus terrestris, Helix pomatia, Locusta migratoria) were killed and their alimentary canals quickly removed, then processed by using different histochemical methods. Before or after the histochemical reaction wholemounts were made by removing the mucosal and submucosal layer. Preparations were mounted on slides in glycerin or liquid paraffin, covered with cover slip and photographs were taken. For the NBT-NADH method the pieces of intestines were incubated in a reaction mixture containing NBT and NADH. Cell counting was made with the help of ocular micrometer directly from the wholemounts. The sizes of cells were measured from photographs. The presence of fluorogenic monoamines was investigated by immersing the tissue in a sucrose-phosphate-glyoxylic acid solution (Fekete, 1984). Some animals were treated with 6-OHDA prior to sacrifice (Fekete, 1986). Immunohistochemical procedure was carried out by the methods of Llewellyn-Smith et al. (1985). An antiserum to GABA was raised in rabbits, characterized by M. Eckert in Jena. The antiserum was used at a dilution of 1:5000. AChE activity was investi-

gated by a modified version of the method of Koelle and Friedenwald (1949).

RESULTS

Table 1 shows that a considerable number of NADH-labelled nerve cells were localized to the pharyngo-oesophageal region of the gut in earthworm. Only solitary cells were seen (Fig. 1), they never grouped into ganglion-like structures. Two types of cell were identified, a relatively large, multipolar cell (Fig. 2) and a smaller uni- or bipolar neuron (Fig. 1, arrow).

Table 1. Results of cell counting and measuring in different invertebrates after NBT-NADH reaction. Maximal profile means the multiplication of the shorter and larger diameter of the cells. Of each animal 100 measurements were made

Species and regions	Max. profile of neurons (μm^2)		Average cell number ($cm^2 \pm SD$)
	small	large	
Lumbricus			
pharynx	10-20	80-100	545±38
Locusta			
coecum	60-70	none	104±12
Locusta			
hindgut	60-70	none	234±23
Helix			
foregut	100-130	800-1000	no data

Smaller and larger (Figs 3, 4, 5) solitary cells were seen in the whole length of the alimentary canal in Helix pomatia. Besides these cells, heavily stained nerve bundles were also seen (Fig. 5). The large-type cells sent their processes to these thick nerve bundles (Fig. 5). The majority of neurons in the snail's gut seemed to be unipolar, with a large, round-

Fig. 1

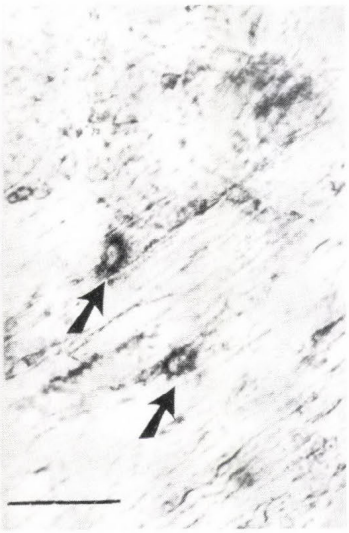


Fig. 2

Fig. 1. Small NADH-positive neurons (arrows) in the pharyngeal region of Lumbricus. Scale bar: 100 µm

Fig. 2. Large sized neurons (arrows) in the pharyngeal region of Lumbricus. Scale bar: 100 µm

shaped cell body (Fig. 3), although some bi- and multipolar neurons from both the smaller and the larger types of cells were also detected (Fig. 4).

Solitary and rarely grouped cells were observed in the hindgut and coecum of Locusta migratoria (Fig. 6). These cells were both bi- and multipolar. Their sizes were uniformly small and they spread all over the coecum. The alimentary canal had large extrinsic nerves, heavily labelled by NADH (Fig. 7). These nerves were always accompanied by tracheas (Fig. 7). In the hindgut of Locusta only multipolar neurons were seen, forming smaller groups of cells (Fig. 8). The whole length of insect gut was intensively supplied with tracheas.

The use of GIF technique on wholemount strech preparates of gut musculature in Helix pomatia and Lumbricus terrestris revealed that part of the intramural neurons handled with catecholamines, showing a characteristic green fluorescence. A rich green fluorescence was seen in the foregut of snail

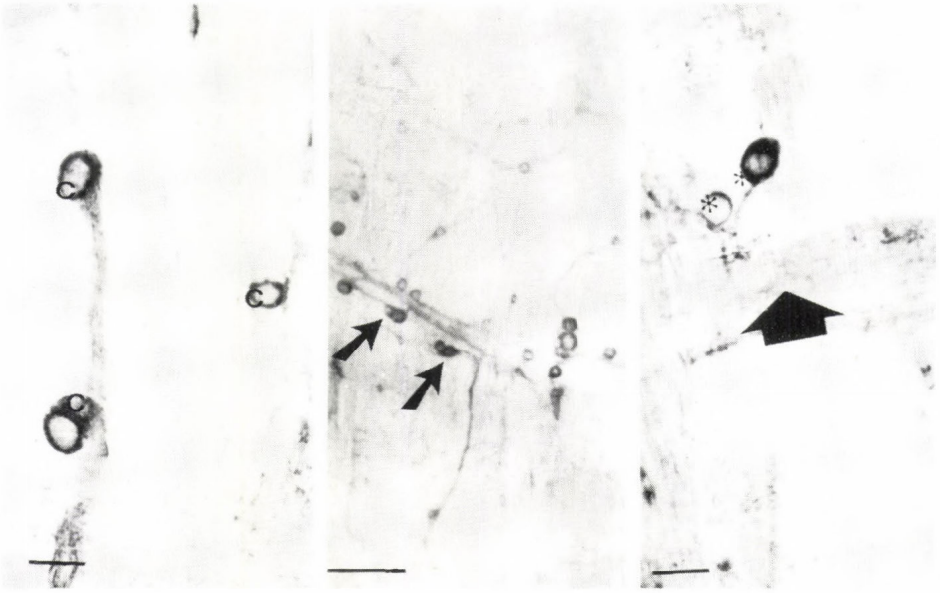


Fig. 3 Fig. 4 Fig. 5

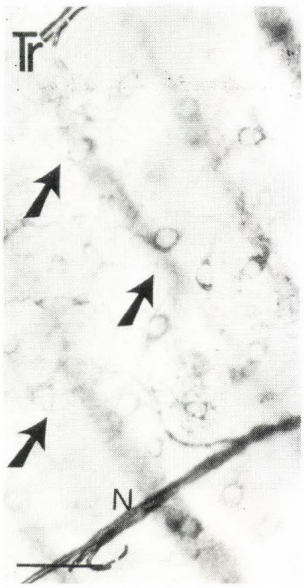
Fig. 3. Large sized, NADH-labelled cells (c) in the midgut of Helix pomatia. Scale bar: 100 µm

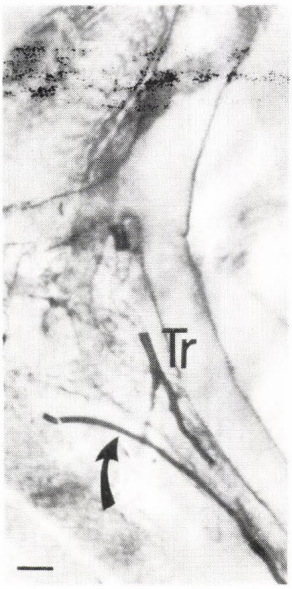
Fig. 4. Small bi- and multipolar cells (arrows) from the hindgut of Helix. Scale bar: 100 µm

Fig. 5. Thick nerve bundles labelled by NADH (arrowheads) in the oesophagus of snail. Connected neurons (asterisk) are seen. Scale bar: 100 µm

(Fig. 9). The fluorescent fibres occurred singly or in bundles and were concentrated in several areas (Fig. 9). A rare occurrence of intensely fluorescent cell bodies of different sizes and shapes was also seen. These cells were either multi-, bi- or unipolar in type. Thin bundles with weak fluorescence were seen in close association with multipolar cell bodies in the stomach, while reactive cell bodies were not seen in the hindgut of Helix pomatia.

On the effect of 6-OHDA administered intracardially (2.5 mg/animal) most of the fluorescence disappeared (Fig. 12). The rest of the fibres showed an irregular distribution and the intensity of fluorescence was reduced. All of the remaining fibres were varicose in appearance (Fig. 13). The number of





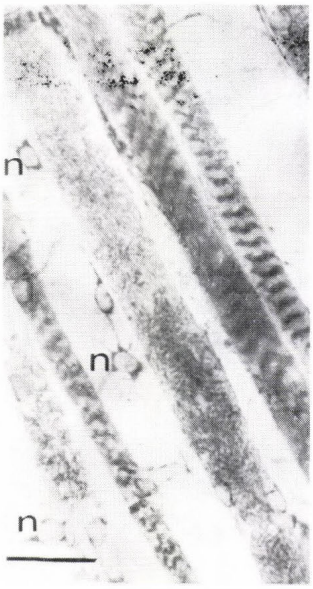


Fig. 6 Fig. 7 Fig. 8

Fig. 6. Small sized neurons are scattered in the coecum of Locusta (Tr: trachea, N: nerves). Scale bar: 100 µm

Fig. 7. Large extrinsic nerves (arrowhead) in the hindgut of Locusta, accompanied by tracheas (Tr). Scale bar: 100 µm

Fig. 8. Typical multipolar neurons (n) in the hindgut of Locusta. Scale bar: 100 µm

fluorescent nerve elements was highly reduced during winter, when the snails suspend feeding completely.

Glyoxylic acid induced fluorescence was localized in the pharyngo-oesophageal region of Lumbricus. Nerve bundles with heavy fluorescence (Fig. 14) and also cell bodies of different shape and size (Fig. 15) were seen in the oesophagus while faintly fluorescent fibres and a few bright cells were seen in the pharynx (Fig. 16).

Besides the aminergic character of the intestinal nerve elements a very intensive cholinesterase positivity was revealed in the midgut of Helix pomatia. A meshwork of AChE positive nerves (Fig. 17) and also several nerve cell bodies connected to these nerves were revealed (Fig. 18).

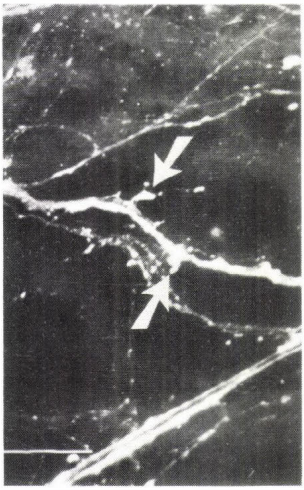


Fig. 9

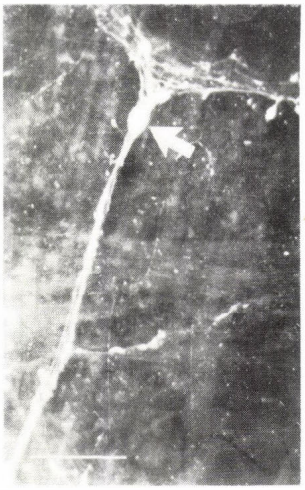


Fig. 10

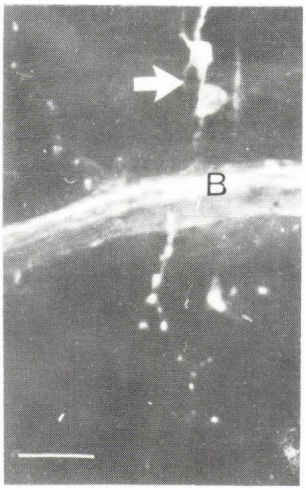
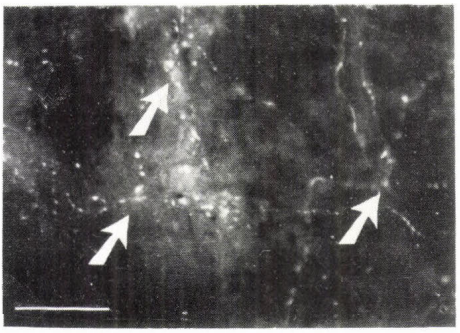


Fig. 11



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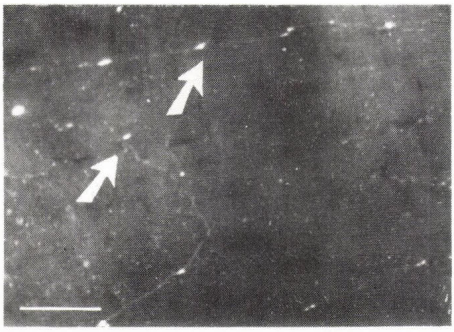

Fig. 13

Fig. 9. Amine-containing nerve fibres and associated cells (arrows) in the midgut of Helix pomatia. Scale bar: 100 µm

Fig. 10. Large cell (arrow) with an intensive green fluorescence in the foregut of Helix. Scale bar: 100 µm

Fig. 11. Nerve bundles (B) and large cells in the foregut of Helix, after glyoxylic acid induction. Scale bar: 100 µm

Fig. 12. Faintly fluorescent nerve fibres after 6-OHDA treatment in the foregut of snail (arrows). Scale bar: 100 µm

Fig. 13. Fluorescent varicose fibres in the foregut of snail in winter. Scale bar: 100 µm

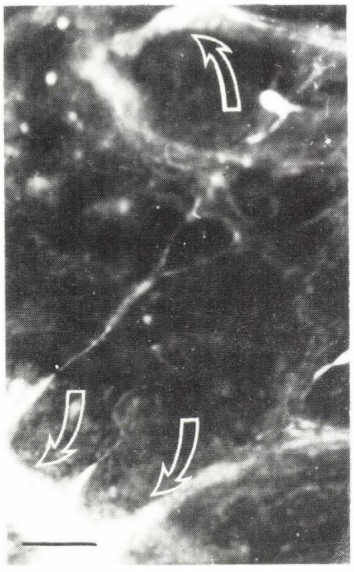

Fig. 14

Fig. 15

Fig. 16

Fig. 14. GA-induced fluorescence in the oesophagus of Lumbricus terrestris. Arrows show the thick nerve bundles with amine-specific fluorescence. Scale bar: 100 µm

Fig. 15. Small intensively fluorescent nerve cells spread out in the oesophagus of Lumbricus terrestris. Scale bar: 100 µm

Fig. 16. Faintly fluorescent nerve fibres in the pharynx of Lumbricus. (N: neurons) Scale bar: 100 µm

During our preliminary immunohistochemical studies we were able to find several GABA positive cells of different shape and size (Figs 19, 20) in the foregut of Helix pomatia.

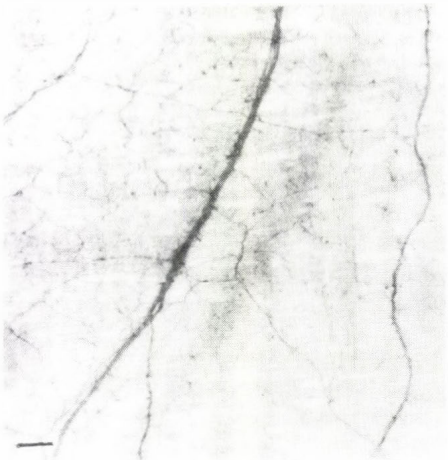

Fig. 17

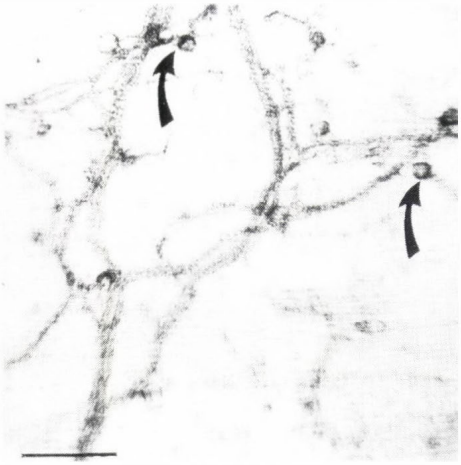

Fig. 18

Fig. 19

Fig. 20

Fig. 17. Fine meshwork of AChE-positive nerves in the stomach of Helix pomatia. Scale bar: 100 µm

Fig. 18. Round AChE-positive cells (arrows) connected to the nerve bundles in Helix. Scale bar: 100 µm

Fig. 19. GABA-positive cells in the foregut of Helix pomatia. Scale bar: 100 µm

Fig. 20. GABA-positive cells in the foregut of Helix of a multipolar character. Scale bar: 1000 µm

DISCUSSION

In the present study we have been able to visualize directly the intramural nerves and their projections in three different invertebrate species with different histochemical methods. We demonstrated that the organization of the enteric nervous system in invertebrates is basically different from that

of higher vertebrates. Namely no sign of ganglionated arrangement can be seen at this phylogenetical level. However, it is very probable that the chemical structure of the enteric nervous system is much more conservative. Our evidence suggests that the nerve elements in the alimentary canal of invertebrates handle with the same transmitters as in higher vertebrates. Based on our histochemical data we suggest that the majority of the catecholaminergic neurons of the studied invertebrate enteric nervous systems can be localized in the foregut, while the enteric neurons in the lower part of the alimentary canal use mainly other types of transmitter substances. Kristján et al. (1986) reported the first direct demonstration of GABA-ergic cells in the enteric nervous system. The fact that we also found several GABA-positive cell bodies in the intestine of snail suggests the possibility that GABA is present in the nerves of the gastrointestinal tract of different phylogenetical groups, too. Our main purpose in the near future is to collect more evidence for this suggestion and also to determine the role of these cells in the neural control of gut function

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DISCUSSION

ELEKES, K.: Did you identify serotonin-containing elements in the enteric nervous system? It is quite possible that more elements fluorescing after 6-OHDA treatment contain 5-HT.

FEKETE, É.: The glyoxylic acid induced fluorescence method we used is not suitable for distinguishing between catecholamines and indolamines in the enteric nervous system, however, the sharp decrease of fluorescence after 6-OHDA treatment allows us to conclude that most of the fluorescence is due to catecholamines.

ELOFSSON, R.: You referred to the intestinal nerve net as primitive. What is the meaning of that?

FEKETE, É.: I referred to the low level of phylogenesis, where the organization of the enteric nervous system is very simple, no ganglia can be seen.

LUKOWIAK, K.: 1. Do the GABA cells project back from the enteric plexus to the CNS?

2. Janet Richmond has shown the presence of FMRFamide and ACT-like neurons in the plexus of Helisoma gut. They appear to project back to CNS.

FEKETE, É.: The whole mount preparations we used did not allow us to follow the cell projections to the CNS.

PENZLIN, H.: What do you think about the nature of the neurons you found in the enteric nervous system? Are they exclusively sensory?

FEKETE, É.: Literary data concerning this question are contradictory. Cytochemical methods alone are not suitable to prove the sensory or motoric nature of these cells.

Symposia Biologica Hungarica 36 (1988)

ULTRASTRUCTURAL COMPARISON OF ENTERIC NEUROMUSCULAR JUNCTIONS IN SOME INVERTEBRATES

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ABSTRACT

The different forms of neuromuscular contacts were studied in the gut wall of earthworm (Lumbricus terrestris), snail (Helix pomatia) and locust (Locusta migratoria) by means of electron microscopy. The nerve terminals in the musculature of the earthworm gut originate from thick nerve bundles situated between the blood sinuses and the obliquely striated muscular layer. The nerve bundles are covered by glial processes, but, at places, the varicosities are free. The gap between the axolemma and sarcolemma is wide (about 200 nm). Neuromuscular synapses have not been found yet. Morphologically two types of nerve terminals were distinguished: one of them contains mainly small agranular vesicles (av.dm. 50 nm) and some dense-cored vesicles; the other is filled with large electron dense granules (av.dm. 200 nm). The nerve supply of snail gut is very dense. Thick nerve bundles, network of thin fibres and neurons can be found in the muscular layer. Close contact is the exclusive form of neuromuscular junctions. The gap between the axolemma and sarcolemma is 15-20 nm. Two types of nerve profiles establish this contact: varicosities with large opaque granules (av. dm. 150-200 nm) and others with dense-cored vesicles (av. dm. 100 nm). Non-synaptic release sites of these terminals were revealed by TARI-method. Nerve cells containing the above

types of granules and vesicles were also frequent in the gut wall. The striated musculature of locust gut receives both non-synaptic and synaptic innervation. Nerve profiles with neurosecretory-like granules (av. dm. 130-200 nm) or those with dense-cored vesicles (av. dm. 100 nm) establish close contact with the striated muscle fibres. At the same time synaptic contacts with well-defined pre- and postsynaptic membrane thickenings are also common. The presynaptic element of these junctions always contains small agranular vesicles (av. dm. 50 nm) besides large (av. dm. 150 nm) granular vesicles. The postsynaptic part of the synapses is rather a process of muscle fibre than the fibre itself. Our morphological results show that the close contact is an overall occurring characteristic form of autonomic neuromuscular junctions, being present in the musculature of the studied invertebrate species. At the same time the striated musculature of invertebrate alimentary tract needs synaptic input too.

INTRODUCTION

Peristalsis results in cranio-caudal transport of nourishment in the alimentary tract of both invertebrates and vertebrates. This movement takes place by means of muscular tissues, which are - in spite of the similar motility - morphologically very variable. For example obliquely striated musculature is characteristic of the alimentary tract in worms; a peculiar smooth muscular layer is in the gut wall of molluscs; at the same time the muscle fibres of insect intestine are cross-striated. A complicated system of intrinsic and extrinsic innervation makes possible the regular peristaltic movements of the mammalian gastrointestinal tract (Burnstock, 1986; Gabella, 1979; Gershon, 1981). First of all the intrinsic part - the enteric nervous system - is responsible for this peristalsis (Gershon, 1981; Langley, 1921; Trendelenburg, 1917). The enteric nervous system of mammals is the result of a phylogenetic development and its

major steps are not negligible. Therefore the aim of this study is to reveal differences and similarities in the fine structural organization of neuromuscular connections in some invertebrates, and to offer in this way a proper basis for further biochemical and physiological studies.

MATERIALS AND METHODS

Studies were carried out on adult earthworms (Lumbricus terrestris), snails (Helix pomatia) and locusts (Locusta migratoria). The animals were killed, the alimentary tracts quickly removed and fixed in 4 % Karnovsky-fixative or 2,5 % glutaraldehyde for 4 hours at 4^{o}C. The tissue-blocks were then washed in 0,2 M phosphate buffer containing 7,5 % sucrose for 10 minutes. After 2 hours postfixation in 2 % osmium tetroxide the material was dehydrated in ascending ethanol series, and embedded in Durcupan ACM. Double staining was performed en bloc with uranyl acetate and on sections with lead citrate. Electron micrographs were taken on JEOL 100 C electron microscope.

For the demonstration of non-synaptic release sites the method of Buma et al. (1984) was used on the gut pieces of snail. Briefly, the tissue blocks were incubated for 1 hour at 20 ^{o}C in Ringer solution containing 0,5 % tannic acid, then fixed overnight in 1 % cacodylate-buffered glutaraldehyde at pH 7, and processed by the conventional method of embedding (see above).

RESULTS

Earthworm

The most primitive form of nerve-muscle junction is characteristic of the earthworm gut: varicosities of nerves are situated at a distance of about 200 nm from the obliquely striated muscle cells and their surface facing the muscle is free of glial cover (Fig. 1). Their vesicle-content can be mixed: small agranular vesicles occur together

with dense-cored ones (Fig. 1: T_1), or the axoplasm contains large granules of peptidergic character (Fig. 2: T_2). The origin of these nerves is mainly extrinsic, the myenteric plexus situated between the muscular layer and the blood sinuses is built up by the above nerves and glial processes, and intrinsic neurons have not been observed electron microscopically until now. The size of the small agranular vesicles is about 50 nm, while the peptidergic granules are much larger, their average diameter is 200 nm. The content of these vesicles and granules can affect the muscle layer through a wide space; although the only sign of the synaptic specialization is the vesicle accumulation and the lack of glial cover at these sites, neither pre-, nor postsynaptic membrane specializations are recognizable.

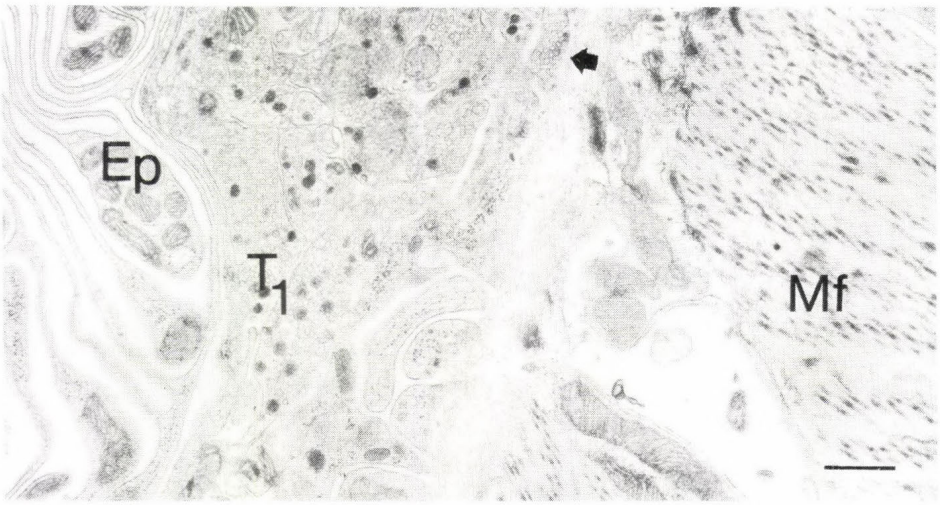

Fig. 1. Neuropil between the blood sinus epithel (Ep) and muscular layer (Mf) in the earthworm oesophagus. The majority of the varicosities (T_1) contains small agranular vesicles and large dense-cored vesicles together. The arrow shows naked surface of an axon profile. Scale bar: 0,5 μm.

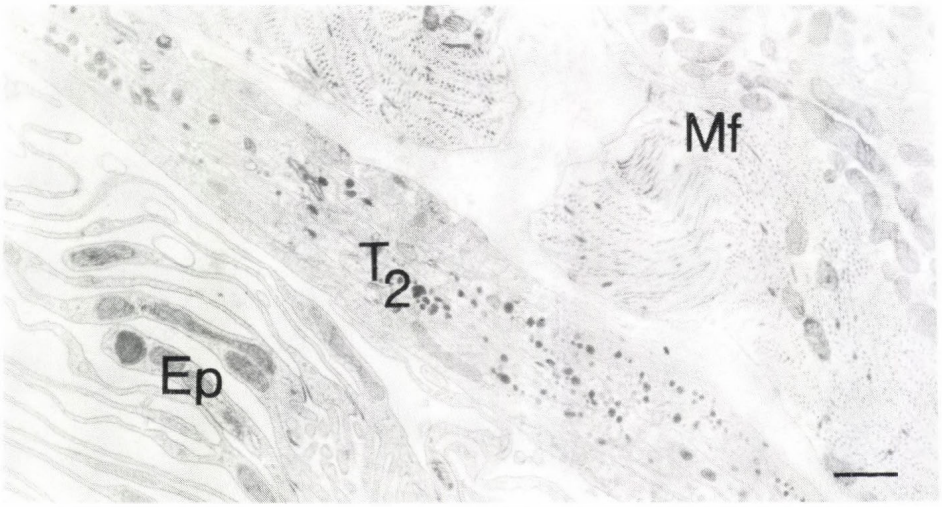

Fig. 2. An elongated nerve plexus in similar position as in Fig.1. Some of the varicosities (T_2) are filled with granules of peptidergic character. Mf = obliquely striated muscle; Ep = blood sinus epithel. Scale bar: 0,5 µm.

Snail

True close contacts with a 15-20 nm wide gap are to be found in the muscular layer of snail. The neural element of the junction can be an axon terminal filled with neurosecretory-like granules, or dense-cored vesicles. The first type (Fig. 3: T_1) of nerve profiles contains granules of varied electron density with an average diameter of 150-200 nm. The other (Fig. 4: T_2) is filled with dense-cored vesicles, their average diameter is about 100 nm. The muscular element is the smooth muscle cell, but no sign of postsynaptic specialization is visible on the sarcolemma. The release of the vesicle content takes place by exocytosis, which is well detectable after TARI-incubation (Fig. 5, A, B). Nerve cell bodies with granules and vesicles of the above character were also frequent in the muscular layer.

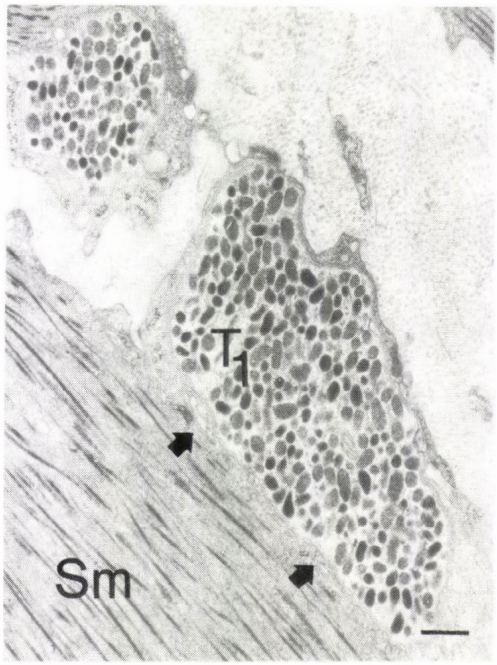

Fig. 3: Axon terminal (T_1) of peptidergic character in close contact (arrows) with the smooth muscle cell (Sm) of snail midgut Scale bar: 0,8 µm.

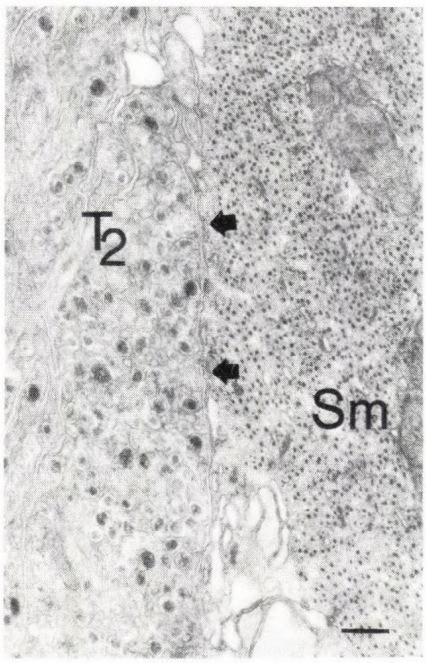

Fig. 4: Axon terminal (T_2) with dense-cored vesicles tightly fits (arrow) to a smooth muscle cell (Sm) in the snail midgut. Scale bar: 0,2 µm.

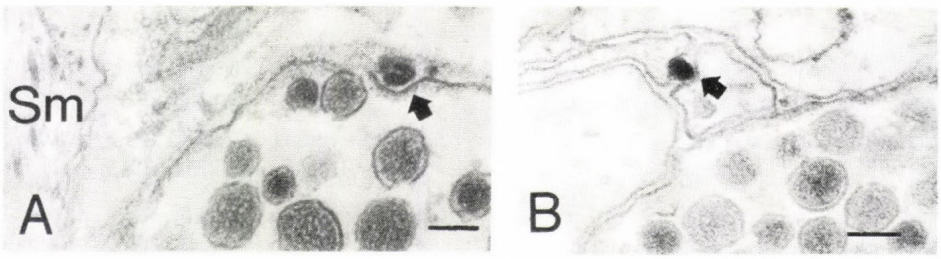

Fig. 5. A, B: Demonstration of no -synaptic release (arrow) in the snail gut after TARI-treatment. Scale bar: 0,1 µm.

Locust

Close contact between nerve terminals and striated muscle fibres occurs in the hindgut of locust too. Mainly peptidergic terminals (Fig. 6: T_1) with large dense granules (average diameter 130-200 nm) establish this type of contacts, however, neuromuscular synapses are also very frequent. The synaptic terminals always contain small agranular vesicles (Fig. 6: T_2) and different granules of variable density. All the morphological features of the classical synapses are present, namely vesicle accumulation, pre- and postsynaptic membrane thickenings (Fig. 6: arrow). The muscle fibres send their processes to the nerve bundles and these processes represent the postsynaptic site (Fig. 6, Fig. 7: P).

Fig. 6: Non-synaptic (T_1) and synaptic (T_2) nerve terminals in the locust hindgut. The arrow shows a neuromuscular synapse. P = muscle process. Scale bar: 1 µm.

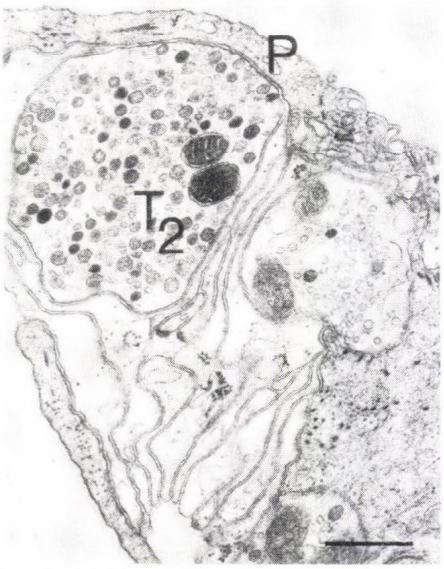

Fig. 7: Intimate nexus of nerve elements (T_2) with the processes of striated muscle fibre (P). Scale bar: 1 µm.

DISCUSSION

The motility of the invertebrate gastrointestinal tract - similarly to the vertebrates - is under intrinsic and extrinsic neural control (Anderson and Cochrane, 1978; Halasy and Benedeczky, 1984; Millott, 1943).

Extrinsic nerves predominate in the alimentary tract of earthworm. These nerves coming from the central nervous system belong to physiologically different groups referring to the sympathetic and parasympathetic system of vertebrates (Millott, 1943). Their relation to the muscle cells is special, and - due to the extremely wide synaptic cleft - very probably represents an early form of nerve-muscle contacts. Contacts similar to our T_1-type were found in the body musculature of other Annelids too, and these were described as cholinergic junctions (Farnesi and Vagnetti, 1975).

Molluscs represent a higher level in the phylogenetic development. The nerve plexus situated in the muscular layer of the snail gut resembles the myenteric plexus of lower vertebrates (Halasy and Benedeczky, 1984). The appearance of intrinsic neurons, the extensive neuropil in the gut wall and the close contacts between the nerve terminals and smooth muscle cells can be evaluated as the marks of a more advanced stage of enteric nervous system.

The different developmental way resulted in different structure and innervation in the locust gut. Here the role of extrinsic neural elements is stressed (Anderson and Cochrane, 1978). The occurrence of synaptic neuromuscular junctions is a unique feature among the studied invertebrate species (Benedeczky and Miller, 1983).

The heterogeneous ultrastructural picture of the nerve terminals taking part in the innervation of the enteric muscles in the studied species suggests a diversity of their neurotransmitter-content. The axon terminals containing large electron-dense granules are processes of neurosecretory cells extrinsic, or intrinsic to the gut. These are present in the gastrointestinal tract of all the three studied species. Their granules usually contain neurosecretum of peptide-nature

(Raabe, 1982), which can affect the function of muscles through non-synaptic release, that is, exocytosis (Benedeczky and Miller, 1983; Buma et al., 1984). Proctolin, a neuropeptide characteristic of arthropods, was found in the insect gut too (Brown, 1975; Eckert et al., 1981). FMRF-amide is a tetrapeptide occurring in the central nervous system of snails (Price and Greenberg, 1977), but other neuropeptides identified first in vertebrates are also detectable in the molluscan central nervous system (Osborne and Dockray, 1982; Osborne et al., 1982). Their presence in the gastrointestinal tract is also very probable. Acetylcholine can be considered as a general excitatory transmitter, except arthropods, where glutamate plays a similar role (Usherwood, 1969), at the same time gamma-aminobutyric acid is very probably the general inhibitory transmitter of invertebrates (Gerschenfeld, 1973).

The presence of different monoamines was proved by histofluorescence in the oesophagus of earthworm (Fekete, personal communication). Not only histofluorescence, but biochemical measurements also have revealed serotonin, dopamine, norepinephrine and epinephrine in the locust and snail gut (Fekete, 1984; Nemcsók et al., 1986; Vig et al., 1985).

Summarizing our results we consider that the enteric innervation of the snail seems to represent the highest degree of organization among the studied species. Similarities with the vertebrate enteric nervous system, namely the presence of smooth muscles, close contacts and intrinsic neurons, support our conclusion.

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DISCUSSION

BRÄUNIG, P.: On one of your last slides of the locust intestine there was adjacent to one of the nerve terminals a structure that reminded me of dendritic profiles of multipolar mechanoreceptors as they are known from insects and crustacea. Do you think that some of the nerve terminals you showed may modulate sensory cells of the gut rather than its muscle fibres?

HALASY, K.: Most of the terminals lying beside muscle cells seem to be axon terminals and not dendritic profiles, because they always do contain a large amount of vesicles. Certainly the presence of sensory dendrites is also possible.

ELEKES, K.: 1. How could you compare your results obtained in the locust gut to those of Klemm et al. (1968)? Namely, they have described one type of terminal forming specialized ring-contacts and two other ones contacting the muscle fibres without membrane specializations?

2. Why do you think so definitely that all of the granules with large diameter are peptidergic?

3. Did you find axo-axonic synapses in the neuropile-like regions of the enteric plexus of Helix gut?

HALASY, K.: 1. We have found at least two types of synapsing and two other non-synapsing types of terminals, as it was described by Benedeczky and Miller (1983).

2. At present we do not have direct evidence about the transmitter content of morphologically different nerve terminals. The general morphology suggests the phrases. I must apologize for the free usage.

3. We have not been able to detect axo-axonic synapses, very rarely axo-somatic contacts occur in the Helix gut neuropile.

MOFFETT, S.: Do you think the muscle cells are the only targets of the enteric neurons, or could the gland cells also be controlled by this system?

HALASY, K.: The enteric plexus is situated between the relatively thin muscular and the epithelial layers in each studied species. It is very probable that not only the muscular layer, but the epithelial layer, too, is under the control of nerves coming from this plexus.

ROUBUS, E.W.: Some terminals seem to make direct contact with the muscle cells, others are located at some distance. Is a basal lamina always present between a terminal and a muscle cell? This might tell us something about the nature (synaptic or diffuse) of the chemical communication at such sites.

HALASY, K.: The basal lamina is present in the case of wide junctional gaps only. The chemical messengers affect the target cells very probably by diffusion.

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CENTRAL NERVOUS SYSTEM REGENERATION IN THE SNAIL MELAMPUS BIDENTATUS AS REVEALED BY SEROTONIN IMMUNOHISTOCHEMISTRY

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INTRODUCTION

The potential for regeneration of the nervous system is present in varying degree throughout the animal kingdom. In gastropod molluscs, sprouting of injured neurons and reestablishment of appropriate connectivity often restores normal behavior following injury to the nervous system (Murphy and Kater, 1978; Janse et al., 1979). Gastropods have been good subjects for the investigation of regeneration following injury to the nervous system because alterations in the anatomy and connectivity of identified neurons have been accessible for analysis (Bulloch, 1985; Allison and Benjamin, 1985; Murphy et al., 1985). We have been studying a pulmonate snail, Melampus bidentatus, that has the ability to repair central pathways and replace central neurons following cerebral ganglion removal (Moffett and Austin, 1982; Moffett and Snyder, 1985; Moffett and Ridgway, 1988).

Our earlier observations on nervous system regeneration in Melampus have raised more questions than they have answered. In the work reported here we have attempted to answer some of these questions, including the degree to which nerve regeneration results from ingrowth of axons from peripheral sensory cell somata, how long distal portions of severed axons survive, how growing axons are guided, whether novel growth of the remaining neurons may compensate for neuron loss, and whether identifiable neurons are regenerated. We have approached these questions by examining how removal of the left cerebral ganglion affects the morphology of the serotonergic cells in other ganglia and the fate of processes that projected from, into or through the missing ganglion.

MATERIALS AND METHODS

Sources of the animals, their maintenance and our surgical methods were as previously described (Moffett and Snyder, 1985). Data were collected from snails sacrificed at intervals ranging from one day to over a year following left cerebral ganglionectomy. The immunohistochemical procedures for wholemounts of the nervous system are detailed in Ridgway (1987). The antibody to serotonin (ImmunoNuclear Corp., Stillwater, Minnesota, USA) was generated in rabbits against a conjugate of serotonin and bovine serum albumin (coupled via formaldehyde) by the method of Steinbusch et al. (1978). We performed specificity controls to confirm that this antibody has a high affinity for serotonin (Ridgway, 1987); however, since the actual antigen is thought to be a tetrahydro-β-carboline 5-hydroxytryptamine derivative (Tandler et al., 1986), we refer to the cells stained by our procedures as having serotonin-like immunoreactivity.

RESULTS AND DISCUSSION

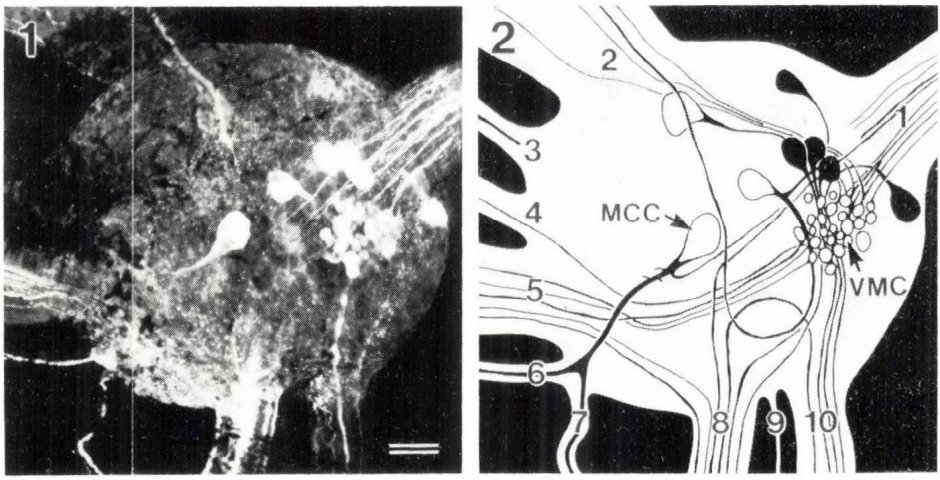
Background

The nervous system of Melampus is primitively unfused, with relatively long connectives linking the pleural, pedal, and buccal ganglia to the cerebral ganglia. Our observations on the sequence of growth following ganglion removal confirms the description given by Price (1977): the median labial nerve serves as a focus for the unification of the other nerves, the connectives, and the cerebral commissure.

Regeneration following cerebral ganglionectomy can be divided into tract, bud, and ganglion stages (Price, 1977; Moffett and Austin, 1982). The unification of the neural tracts that extend through the site of the missing ganglion is typically complete within two months and regeneration proceeds to the ganglion bud stage. A discrete or elongated swelling develops and continues to enlarge in subsequent months. The final stage, ganglion (neuron) replacement, is seen in roughly half of the snails examined six months or longer after ganglion removal. Neurons are detectable under the sheath in clusters or as a continuous rind overlying the neuropil region (Moffett and Austin, 1982; Moffett and Snyder, 1985; Moffett and Ridgway, 1988).

In the left cerebral ganglion of control snails, between 30 and 35 serotonin-like immunoreactive (SIR) neurons are present (Figs 1 and 2). These include a metacerebral cell (homologous to the "metacerebral giant cells" of other gastropods), other individual neurons and clusters

containing medium-sized or small neurons. The individual neurons and neuron clusters have been described, along with details of their axonal projection pattern (Ridgway, 1987).



Figs 1 and 2. Serotonin-like immunoreactive cells in the left cerebral ganglion. Drawing in Fig. 2 gives details obtained from focusing through the ganglion shown in Fig. 1. Filled neuron somata are dorsal; unfilled somata are ventral. Incoming axons as well as projections from the ganglion cells are indicated. MCC: metacerebral cell; VMC: ventromedial cluster; nerves and tracts numbered: 1, cerebral commissure; 2, tentacle nerve; 3, optical nerve and cerebral tube; 4, anterior labial nerve; 5, median labial nerve; 6, arterial labial nerve; 7, cerebrobuccal connective; 8, cerebropedal connective; 9, statocyst nerve; 10, cerebropleural connective. Bar = 50 µm.

Serotonin immunohistochemistry of tract and bud stage regenerates

Regeneration in the early period of tract formation can best be described by examining the growth in three regions that are initially involved in bridging the gap created by removal of the left cerebral ganglion. These regions are (1) the cerebropedal and cerebropleural connectives, (2) the labial nerves, and (3) the cerebral commissure.

In the first few days following ganglion removal, the most striking observation provided by the immunohistochemistry technique is the growth of SIR processes that project through the cerebropleural connective and especially the cerebropedal connective (Fig. 3). In the fused tract formed by regenerating fibers from the two connectives, five to seven

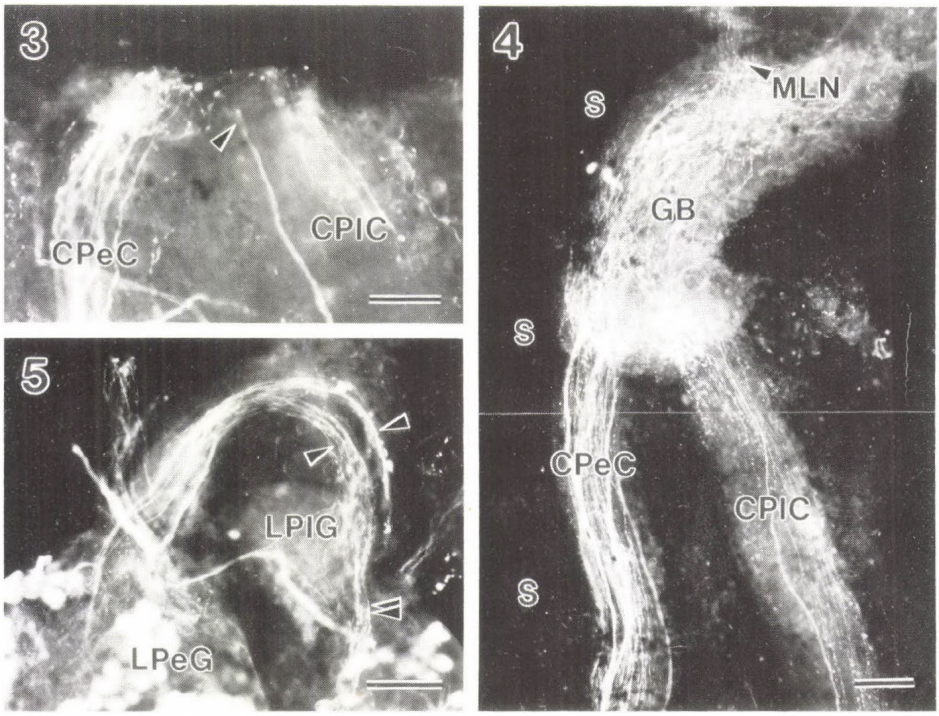


Fig. 3. Three days after left cerebral ganglion removal, growth has occurred at the ends of axons that project through the cerebropedal connective (CPeC) and cerebropleural connective from SIR neurons located in the pedal and other ring ganglia. In contrast, no growth occurs at the end of the distal portion of axons (arrow) that extended from SIR neurons in the ablated left cerebral ganglion. Fig. 4. By 21 days, the fused connectives have become part of the ganglion bud (GB). A continuous sheath (S) provided a linkage between the labial nerves (median labial nerve, MLN) and the connectives. Fig. 5. An example of misdirected axons in a snail sacrificed 14 days after the left cerebral ganglion was excised. Growth from both the left pedal ganglion (LPeG) and left pleural ganglion (LPlG) has formed a loop (arrows). The pedal projections have grown on through the pleural into the left parietal ganglion (double arrow). Bars 3-5: 50 μm, 75 μm, 50 μm.

large axons in the cerebropedal connective are typically joined by at least one axon in the cerebropleural connective; these grow toward the developing junction with the labial nerves (Fig. 4). The SIR projections in the developing bud include both neurites that grow through the tracts and a multitude of tiny varicosities in the sheath that are likely to be transmitter release sites. Such varicosities are commonly reported in

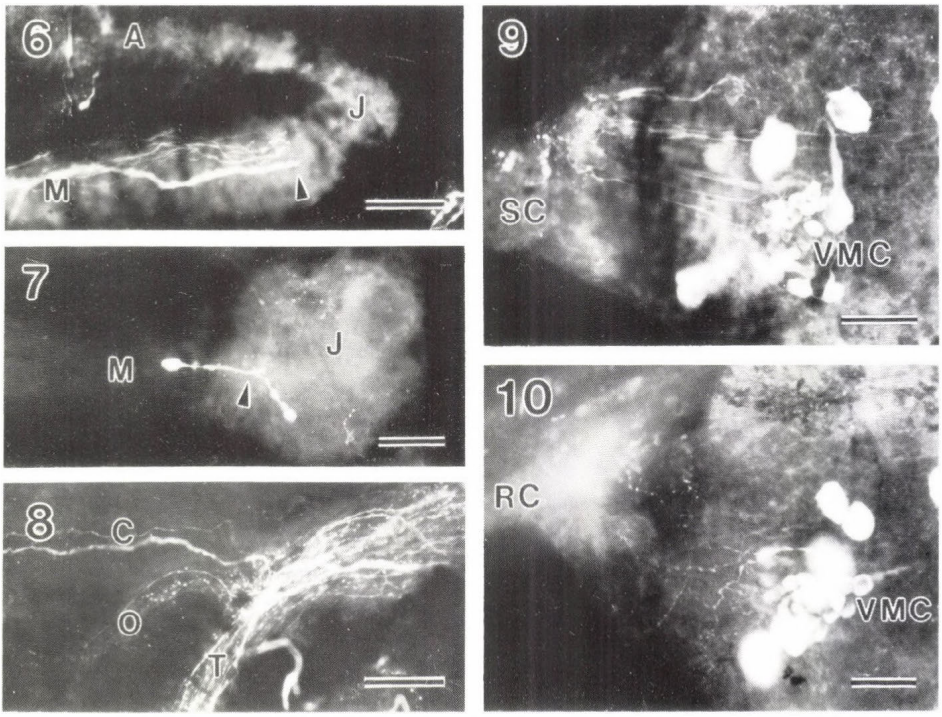


Fig. 6. Three days after ganglion removal, the site of nerve section is marked by distal SIR axon stumps (arrow). Growth of non-SIR neurites has produced a junction (J) of the median lip nerve (M) and the anterior labial nerve (A). Fig. 7. Fourteen days after ganglion removal the last SIR axon (arrow) in the median lip nerve (M) exhibits retraction bulbs and constrictions along the axon. The junction (J) between labial nerves is much more developed than in Fig. 6. Fig. 8. By two months, SIR axons are present in the cerebral commissure (C), which joined the bud near the optic (O) and tentacular (T) nerves. Fig. 9. SIR axons, including those from the ventromedial cluster (VMC) that cross the cerebral commissure from the right cerebral ganglion are shown projecting into the stump of the commissure (SC) three days after left cerebral ganglion excision. Fig. 10. Fourteen days after left cerebral ganglion removal, non-SIR growth contributes to the regenerating commissure (RC) and VMC projections are less apparent. Bars 6-10: 75 μm, 50 μm, 100 μm, 50 μm, 50 μm.

wholemounted gastropod nervous systems (Longley and Longley, 1986, Murphy et al., 1985). We occasionally found axons that did not proceed all the way to the bud region, but instead formed a loop from one connective to the other (Fig. 5). This was most common in instances in which ganglion removal disrupted the connective tissue sheath that normally links the

neural tracts to the ganglia or when ganglion removal severed the connectives close to the pedal and pleural ganglia, increasing the regeneration distance. There is evidence from gastropod regeneration studies in which no neurons have been removed (Bulloch, 1985; Allison and Benjamin, 1986) that axons may produce such loops and grow long distances to access their normal targets by alternative routes; we do not know the fate of the rerouted axons in this case.

In contrast to the growth seen in the axons projecting from neurons elsewhere in the nervous system, distal portions of SIR axons that projected from the extirpated ganglion into the cerebropleural connective did not appear to sprout (Fig. 3) and typically disappeared within 21 days. The disappearance of these as well as other distal axon stumps was occasionally observed to involve a beadlike disintegration pattern suggestive of engulfment by phagocytes (Fig. 7). Phagocytosis by glial cells has been found to occur following lesions to serotonergic axons in the snail Planorbis (Pentreath et al., 1985). Similar patterns of survival and evidence that distal parts of neurons remain functional have been reported by Murphy and Kater (1978) and Murphy et al. (1985) for neurons in the snail Helisoma.

The labial nerves were first described by Price (1977) as the focus for ganglion regeneration in Melampus. These three nerves are closely united by the connective tissue sheath associated with the cerebral ganglia. Centrally-directed growth from these nerves produces confluence of the nerve stumps. The processes forming this junction are not serotonergic and are likely to be sensory fibers with somata in the periphery (Fig. 6). Survival of the distal portions of SIR axons in the labial nerves is apparent for two to four weeks, but there is no evidence of sprouting from these distal axon stumps (Fig. 6). They do survive long enough that their presence might have an influence on growing axons, perhaps through release of a factor that identifies the target site. However, there are likely to be other neuron types that also may be responsible for stimulating and guiding axonal growth (Bulloch, 1987; Bulloch and Jones, 1988).

The first SIR axons seen in the commissure can typically be traced to processes that originated in the left connectives and projected through the ganglion bud and across into the developing cerebral commissure (Fig. 8). Despite their proximity to the commissure, cells of the ventromedial cluster (Figs 1 and 2) as well as other SIR cells in the right cerebral

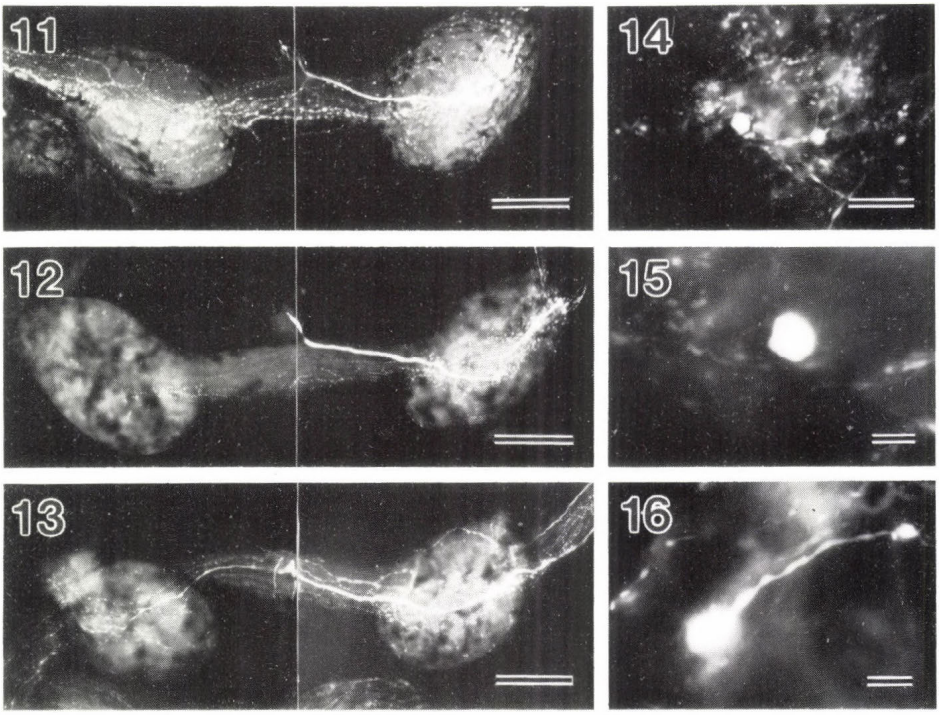


Fig. 11. Control buccal ganglia showing projections of metacerebral cell. Fig. 12. Fourteen days after left cerebral ganglion removal, SIR marking is greatly diminished in left buccal ganglion. Fig. 13. By four months, the right MCG has increased its projections into the left buccal ganglion and on into nerves supplying the left buccal musculature (not shown). Fig. 14. Left cerebral ganglion regenerate (6 months) showing SIR structures. Fig. 15. Higher magnification of area shown in Fig. 14. Fig. 16. Another left ganglion regenerate at 6 months showing small neuron with axonal process. Bars 11-16: 75 μm, 75 μm, 75 μm, 20 μm, 5 μm, 5 μm.

ganglion, are very slow to send projections into the regenerating commissure (compare Fig. 9 and Fig. 10). Only two-thirds of the approximately 25 cells in the ventromedial cluster send axons into the commissure, and these same neurons also have axons in the tentacle nerve. Injury to the commissural projections of these cells may cause retraction of the axon stump while stimulating growth of the tentacular projection. The possibility that removal of the major axon or failure to contact a normal target can result in cell death is suggested by the occasional detection

of fewer than the normal contralateral number of cells in the ventromedial cluster after the left cerebral ganglion is removed.

Even though neurons are regenerated in some snails, behavioral recovery is often so good in snails that do not progress beyond the ganglion bud stage that some form of compensation for the missing neurons is likely. Compensatory growth of new axons by neurons remaining after one cerebral ganglion is removed has been explored by examining projections of the right metacerebral cell into the buccal ganglia. The normal pattern of the metacerebral cell's projections to the buccal ganglia is shown in Fig. 11. Only a very few branches pass over the buccal commissure to the contralateral side. Disappearance of the projection from the left metacerebral cell 14 days after left ganglion extirpation (Fig. 12) reveals the remaining cell's projection pattern. However, in a snail sacrificed four months after left ganglion removal, the axon from the right metacerebral cell extends several novel branches to the left buccal ganglion and through it to the musculature on the left side of the buccal mass (Fig. 13). Thus a degree of compensation has been demonstrated in the absence of a regenerated metacerebral neuron. A considerable body of data exists on the pattern of axonal projections of the "metacerebral giant cells" of gastropods (Granzow and Rowell, 1981; Pentreath et al., 1982) and it is interesting that the compensatory pattern in Melampus corresponds to the normal pattern in some other pulmonates. Extensive novel sprouting of identified neurons has been documented for a pedal neuron in Lymnaea (Allison and Benjamin, 1985) and for buccal neurons in Helisoma (Murphy and Kater, 1980; Bulloch, 1985).

Serotonin immunohistochemistry of ganglion stage regenerates

We were eager to determine if SIR neurons could be identified among the neurons of regenerating ganglia, and if so, whether they appeared early or late in the development of a new ganglion. In most of the early ganglion buds that appeared to have cells on the basis of in situ observation, we found only SIR processes and no immunoreactive somata. In some preparations however, we did detect a few small, round or oval structures that exhibited immunoreactivity (Figs 14 and 15). These SIR structures are 5-7 μm in diameter, several times larger than neuritic varicosities within the bud, and they appear to be cells. The absence of any apparent axon made identification of these cells as neurons quite tentative, but in several other regenerating ganglia, SIR neurons with short axonal

projections were seen (Fig. 16). Examination of regeneration early in the ganglion stage suggests that the SIR neurons are of a class that differentiates after other types of neurons are already present. The observation that early stages of ganglion formation includes SIR cells that have not yet grown axons is of considerable interest in the question of what the transmitter may be doing at this stage of ganglion replacement. Serotonin immunoreactivity does not appear prior to axon extension in developing insects (Taghert and Goodman, 1984), but has been described in neuronal development of sea urchins (Bisgrove and Burke, 1986). The two features of differentiation appear to occur concomitantly in normal ganglion development in Helisoma (J. I. Goldberg, pers. comm.).

Where the first neurons that populate the regenerating ganglion originate was not determined from this study of SIR neurons and processes, but comparison of the numbers of SIR cells in the nervous system of Melampus as a function of snail size (May et al., 1987) has indicated that cell addition occurs in some ganglia of adult snails in the size range that we typically include in our experiments (between 5 and 10 mm shell length). This evidence for addition of neurons in adulthood supports the hypothesis that the ganglion could be "seeded" by migration of either pluripotent stem cells or undifferentiated post-mitotic precursor cells from other ganglia (Ridgway, 1987; Moffett and Ridgway, 1988). We have not seen SIR cells migrating in the commissures and connectives, but this is consistent with our observation that this type of neuron differentiates within the regenerating ganglion after other neurons are already present.

SUMMARY

The comparison of serotonin immunoreactivity in wholemounts of the control and regenerating nervous system of Melampus bidentatus has revealed details of regeneration in this one class of neurons. The sequence of events that we observed in ganglion replacement included early growth by some but not all of the known SIR projections leading into the missing ganglion. During this same time, the distal stumps of severed axons retained immunoreactivity although they did not sprout and eventually disappeared. The projections that normally enter the left cerebral ganglion from the cerebral commissure and the left connectives do not invariably enter the bud, and at least some of these rerouted axons tend to persist for many months. The possibility that ganglion removal can result in the death of neurons in other ganglia was suggested

by counts of SIR cells in the (non-excised) right cerebral ganglion. Compensation for missing neurons by axonal growth of homologous cells was suggested by the projection of the right metacerebral cell into the left buccal ganglion. None of the SIR neurons that we have detected in regenerated ganglia could be identified as a replacement of the metacerebral cell. The projections of this cell's homolog into the left buccal ganglion neuropil and left buccal musculature suggest that the function of this cell can be restored without neuron replacement. The function of the SIR neurons that are regenerated is not yet known, and we do not know the degree to which these observations on SIR neurons can be extended to other classes of neurons, but the techniques applied here have given us valuable insights into the regeneration process in Melampus.

Acknowledgments

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DISCUSSION

BRUSSAARD, A.B.: 1. Did you do comparable studies on animals in which you just crushed the connectives of cerebral ganglia to other ganglia instead of cutting out a whole ganglion?

MOFFETT, S.: Yes, we reported this (Moffett and Snyder, 1985, J. Neurobiol. 16:193-207). Melampus recovers very well after we cut commissures and connectives, and behavioural recovery is correlated with anatomical repair and electrophysiological evidence of pathway restoration.

BRUSSAARD, A.B.: Do you have any suggestion to enhance regeneration?

MOFFETT, S.: I am interested to hear your suggestions.

BRUSSAARD, A.B.: Yes, I have a suggestion. Recently it was shown by the group of Prof. Gispen in Utrecht (Netherlands) that a substance called ORG 2766 (Organon, OSS, Netherlands) enhanced (behavioural and biochemically measured) regeneration, that is: outgrowth of axons in the nerve to the hindleg in rats after crushing the whole nerve.

ELLIOTT, C.J.H.: How long do your snails live in the laboratory?

MOFFETT, S.: About 10 years.

ELLIOTT, C.J.H.: So 6 months of regeneration is a relatively short part of the life-span.

MOFFETT, S.: In the literature the life-span of these snails is indicated as being 3-5 years, but I have maintained snails (that I had operated on as adults) for an additional 5-6 years, so I would guess that they can live as long as 10 years under carefully controlled conditions.

JANSE, C.: You suggested that behavioural recovery after ectomy of one cerebral ganglion is caused by take over of function by neurons in the intact c.g. If this is true one would expect equal threshold of reflective withdrawal upon ipsilateral and contralateral tactile stimulations. In control animals threshold should be lower upon ipsilateral stimulation. Did you observe this?

MOFFETT, S.: I do observe a lower threshold to ipsilateral tactile stimulation in control snails. I have difficulty in generalizing the pathway used for withdrawal responses in regenerated snails, because both contralateral cerebral neurons and neurons in the pedal and other ganglia can participate in this response. In one regenerated snail I did record one-for-one axonal spikes in the ipsilateral and contralateral anterior labial nerve (L_2) and in that same animal the cobalt backfill from the labial nerve on the regenerated side stained a cell in the contralateral cerebral ganglion. As far as the threshold is concerned, many more neurons typically are involved in the response on the intact side, but we have the impression that synaptic contacts are formed in the ganglion bud neuropile between ipsilateral sensory cells and projections of motor neurons that have their somata elsewhere in the CNS. We can occasionally evoke reflex responses in a ganglion bud isolated

from the rest of the CNS, and in which there was no evidence of neuron replacement. So behavioural observations can only indicate the presence but not the precise site of reflex circuitry.

KUTSCH, W.: Is there any correlation between the age of the snail and the potency to regenerate the excised ganglionic pathways?

MOFFETT, S.: We have a feeling that younger adult snails are more successful in regenerating pathways than older snails, but we have not systematically studied this in Melampus. We do know that certain populations of neurons with serotonin-like immunoreactivity increase in number at a more rapid rate in the young adults, and this potential for increase in number of neurons may be related to the ability to add neurons to the regenerating ganglion bud to form a true ganglion.

JANSE, C.: In Lymnaea we showed that regeneration is age related (Janse et al., 1986; Mechs Ageing Dev. 35: 179-183).

LUKOWIAK, K.: 1. Have you any idea as to how you may get growth of new neurons in the regenerating ganglion?

2. You might wish to use the technique developed by Larry Katz. This technique involves the use of microspheres which contain Lucifer Yellow (LY). These are taken up into the axon of any neuron and transported back to the soma of the neuron. Thus it is possible to identify where the cell's innervation is. The LY does not cause photo inactivation when light is shone on it because it is imbedded in the microsphere.

MOFFETT, S.: 1. We feel that there are 3 possible origins of the new neurons: First, from adjacent (non-excised) ganglia, by dedifferentiation of cells, migration of undifferentiated post-mitotic cells or migration of blast cells which subsequently differentiate in the new bud. Second, the new neurons might originate in ectodermal placodes, as has been documented in the development of ganglia in gastropods. A third possible source of new neurons is from the population of haematocytes.

As you saw from our results, it does appear that at least the cells with serotonin-like immunoreactivity are differentiating in situ in the regenerating ganglion.

ROUBOS, E.W.: Melampus is a Basommatophore, so the egg-laying controlling cells will be located in the cerebral ganglia (CDC). What happens to egg-laying and egg-laying behaviour during and after regeneration of a cerebral ganglion?

MOFFETT, S.: Chris Price (1977, Cell Tiss. Res. 180:529-536) reported that egg-laying behaviour was normal after left cerebral ganglion removal. We have not examined this because we maintain the animals on a winter photoperiod, but I presume that the CDC in the remaining right ganglion suffice, and it is possible that this population increases in number following removal of the contralateral ganglion.

SONETTI, D.: Are you going to culture neurons from central nervous system of Melampus? It could be interesting to study in vitro some aspects of regeneration.

MOFFETT, S.: I agree. The factors that stimulate axonal growth and that determine the path taken by regenerating axons and their choice of particular synaptic contacts can best be sorted out in culture. We have been interested in determining the timetable for regeneration in Melampus so that we can focus (in culture) on the neuron replacement phenomenon, which comes much later than the early responses to CNS disruption.

van der WILT, G.J.: 1. What is the role of the connective tissue in neuronal regeneration?

2. Is there a way to influence the neuronal regeneration after removal of one cerebral ganglion?

MOFFETT, S.: 1. The edge of the connective tissue sheath provides a pathway that is preferred by the growing axons. We find that if we greatly disrupt this sheath, which normally unites the nerves and ganglia, the regenerating tracts may become lost

in the periphery. So, we feel that contact adhesion to the sheath is very important in bringing the regenerating axons to the site of bud formation.

2. I am sure that we will find a way to do so, with the neurotransmitters that are being discovered which have neurotrophic or mitotic effects, but we have not yet begun to manipulate the system in this way.

WINLOW, W.: 1. Are there really no behavioural changes following lesions of one cerebral ganglion? For example are there no major modifications to the tentacular withdrawal reflexes?

2. The metacerebral cells are electrically connected to one another in the normal animal. Do non-electrically connected cells send contralateral branches to the ganglionic bud?

3. In Lymnaea there are many well known chemical and electrical synaptic connections between the pedal ganglia and also elsewhere in the visceral and parietal ganglia. Is the Melampus system sufficiently similar to that of Lymnaea or other related gastropods to exploit these known connections?

MOFFETT, S.: 1. I want to stress that it is only in some of the snails that we get good recovery of tentacle retraction behaviour in regenerated snails (Moffett and Snyder, 1985, J. Neurobiol. 16:193-209). Many snails show hyperexcitability in early stages of behavioural recovery, and this can persist for months. It is tempting to speculate that this hyperexcitability may be related to the formation of widespread chemical and electrical connections in response to CNS injury.

2. The question we really need to pose in this study is whether or not some neurons in the intact ganglia develop novel (compensatory) axonal projections in response to ganglion ablation, and whether it may be only injured cells that respond in that way. As you point out, each metacerebral (giant) cell has a projection into the cerebral commissure and is therefore likely to experience axotomy when we remove the contralateral ganglion. We have not yet examined the response(s) of identified neurons that do not experience axotomy in the ganglion ablation.

3. Much work remains to be done in describing synaptic connections between identified neurons in Melampus, but anatomical maps and my preliminary intracellular mapping studies suggest many similarities that will simplify the identification of cells and their connectivity.

PEPTIDERGIC MECHANISMS

Symposia Biologica Hungarica 36 (1988)

STRUCTURAL AND FUNCTIONAL ASPECTS OF THE EGG LAYING CONTROLLING CAUDODORSAL CELLS OF LYMNAEA STAGNALIS

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The peptidergic Caudodorsal Cells (CDC) of the snail *Lymnaea stagnalis* control egg laying and egg-laying behaviour by releasing various peptides that act upon different targets (e.g., Roubos, 1984; Geraerts et al., 1987). Egg laying lasts about 2 hours. Egg-laying behaviour consists of a number of stereotyped behavioural acts, including changes in feeding and locomotory activity, and oviposition (Goldschmeding et al., 1983) (Fig. 1). In this paper a brief survey will be given of the cellular dynamics by which the CDC synthesize and release multiple peptides resulting in coordinated egg laying and egg-laying behaviour. Particular attention will be paid to new data on the structural aspects of CDC functioning.

CDC MORPHOLOGY

The CDC occur in two clusters in the cerebral ganglia (left: ca 25 cells, right: ca 75 cells) (Joosse, 1964; Roubos, 1984). Per cluster ca 7 ventral CDC send an axon branch through the cerebral commissure (Fig. 2). Such "crossing axons" make electrotonic contacts with the contralateral CDC, thereby enabling all CDC to function as one unit (de Vlieger et al., 1980; Roubos et al., 1985). The cells pack their secretory material, including the ovulation hormone CDCH, into electron-dense secretory granules. The granule contents are released into the haemolymph from neurohaemal axon terminals in the periphery of the cerebral commissure (Roubos and van der Wal-Divendal, 1980; Roubos and van de Ven, 1987; Roubos et al., 1987b). In addition, nonsynaptic ("paracrine") secretion occurs into the intercellular space of the cerebral commissure, from nonsynaptic release sites of CDC collaterals (Roubos et al., 1983; Bumâ and Roubos, 1986; Schmidt and Roubos, 1987a,b).

BIOSYNTHESIS OF CDC PEPTIDES

By the use of differential screening methods and synthetic oligonucleotides derived from the primary amino acid sequence of CDCH, various cDNA clones have been elucidated which

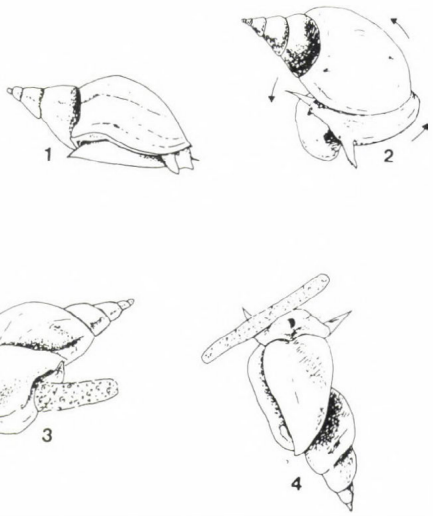

Fig. 1. Postures during the four phases of overt egg-laying behaviour of *L. stagnalis:* 1 resting, 2 turning, 3 deposition, 4 inspection (after Geraerts et al., 1987).

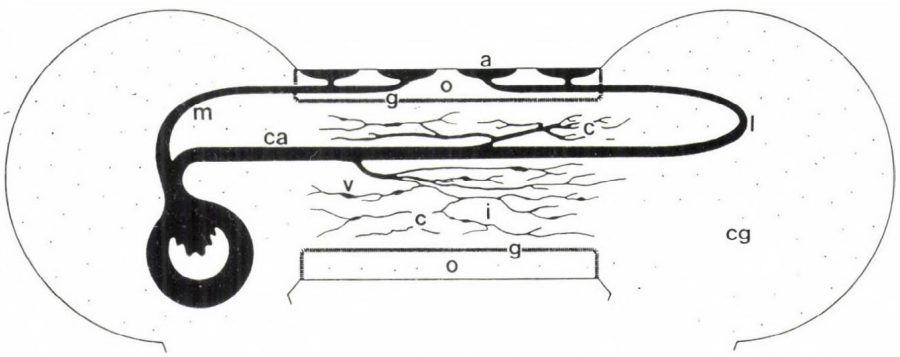

Fig. 2. Outer (o) and inner (i) compartments of the cerebral commissure (COM) of *L. stagnalis*. The compartments are separated by a glial sheath (g). Only one ventral CDC is shown. Its main axon (m) runs through the loop area (l) to the neurohaemal area, the outer compartment, where the axon terminals (a) end blindly. A branch of the main axon, the crossing axon (ca), runs through the inner compartment (i), passes through the contralateral loop area, and then runs to the outer compartment. In the inner compartment the crossing axon gives rise to the collaterals (c). cg cerebral ganglion, v varicosity.

encode CDCH and related peptides (Vreugdenhil et al., 1985; Geraerts et al., 1987). Subsequent Northern blotting experiments demonstrated that the CDC contain various mRNA species encoding different precursors for CDCH(-like peptides) (E. Vreugdenhil, unpublished results) (Fig. 3). One gene encodes the CDCH-precursor, which structure has been completely

sequenced and includes CDCH (Geraerts et al., 1987). Another gene has been partly sequenced; it encodes a CDCH-like precursor, which shows strong homology as well as clear differences with the CDCH-precursor. It contains the sequence of a CDCH-like peptide.

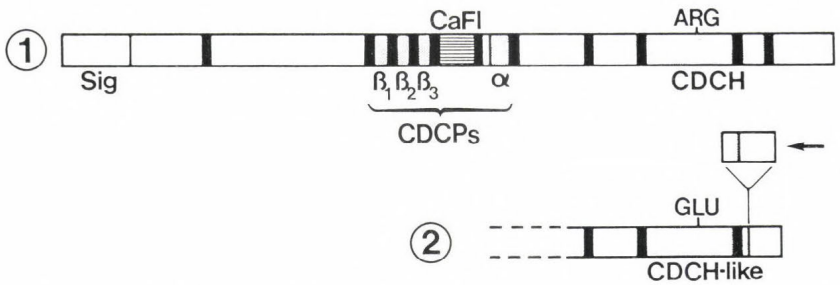

Fig. 3. Structure of the CDCH- (1) and the CDCH-like (2) precursors in the CDC. Potential cleavage sites are indicated by vertical bars, arrow points to deletion. CaFl calfluxin, Sig signal peptide.

On the basis of potential cleavage sites (pairs of basic amino acids) in the precursors a large number of peptides can be assumed to be produced by the CDC, including CDCH, the CDCH-like peptide, αCDCP, three pentapeptides (β-CDCP's) and calfluxin. CDCH consists of 36 amino acids and induces ovulation of egg cells from the ovotestis (Ebberink et al., 1985; Geraerts et al., 1987). Possibly, αCDCP is an auto-excitatory messenger (Moed et al., 1987), which induces high electrical CDC activity (the "discharge"; see below). Calfluxin consists of 14 amino acids (W.J.A.G. Dictus and R.H.M. Ebberink; unpublished results; Mahon et al., 1985) and stimulates the influx of calcium into the mitochondria of the albumen gland *in vitro* (Dictus et al., 1987). The influx may be involved in the stimulation of glandular synthesis and/or release of perivitellin fluid, which surrounds the egg cells.

Pulse-label and pulse-chase studies have indicated that the CDC synthesize a 35 kDa protein that is cleaved into three intermediates (20, 10 and 7 kDa) and 5 end products of 6, 4.5, 3.5, 2 and 1.5 kDa molecular weight classes. The 4.5 kDa class contains CDCH-activity (Geraerts et al., 1985). Probably, the 35 kDa protein is the CDCH- and/or CDCH-like precursor, whereas the other end product classes may represent other CDC peptides.

CDCH contains 6 arginine residues. Ultrastructural radioautographic (Roubos, 1985; Roubos et al., 1987b; E.W. Roubos and W.R.A. van Heumen, unpublished results) and biochemical pulse-chase studies with ^{3}H-arginine (Geraerts et al., 1987) show that CDCH is synthesized within 20 minutes by the rough endoplasmic reticulum (RER) and packed by the Golgi apparatus into secretory granules. The cellular dynamics of CDCH have been studied in some detail using an antiserum raised to a synthetic portion of CDCH comprising the 20-36 amino acid sequence (Roubos et al., 1987a). Specific immunoreactivity (immunogold technique) occurs in all CDC. RER and Golgi apparatus are hardly reactive as are secretory granules that are being formed by the Golgi apparatus. However, secretory granules that occur

at some distance from the Golgi area show more immunoreactivity, whereas mature secretory granules, located distantly from the Golgi apparatus, are strongly reactive. Apparently, CDCH is cleaved from the CDCH-precursor within secretory granules during granule migration from the Golgi apparatus.

Preliminary immunoelectron microscopy studies using poly- and monoclonal antibodies indicate that CDC secretory granules do not only contain CDCH but also αCDCP, the CDCH-like peptide and calfluxin. At least some of the peptides (e.g., CDCH, the CDCH-like peptide and calfluxin) occur within the same granule (Fig. 4).

AXONAL TRANSPORT OF SECRETORY GRANULES

Radioautography indicates that secretory granules in the CDC travel at a speed of 15-20 mm/day (Roubos, 1985; Geraerts et al., 1987). Pulse-label and pulse-chase experiments in the presence of blockers of axonal transport such as colchicine and vinblastine, show an accumulation of intermediates and end products in the somata, and a drastic delay in the appearance of end products in the neurohaemal axon terminals. Ultrastructural observations show that vinblastine, vincristine and colchicine cause a strong accumulation of secretory granules within the CDC somata, whereas no repletion of the neurohaemal area after experimentally induced hormone release occurs (Roubos et al., 1987b; unpublished data). Apparently, this block of axonal granule transport is caused by action upon the microtubular system, which disappears after colchicine treatment, and transforms into paracristalline structures by reacting with Vinca alkaloids (Müller et al., 1987). These data indicate that microtubules are responsible for the transport of the secretory granules in the CDC axons.

NEUROHAEMAL RELEASE

The CDC reveal three states of electrical activity, *viz.* the active state ("discharge"), lasting ca 1 hour, the subsequent inhibited state (ca 5 hours) and, finally, the resting state, which has a variable length and may last up to several days (Kits, 1980). *In vitro* and *in vivo* (fine wire) studies show that the CDC discharge is essential for egg-laying (Kits, 1980; ter Maat et al., 1986). The auto-excitatory peptide plays an important role in the onset of the discharge (Moed et al., 1987). Probably, it acts via receptors upon adenylate cyclase, which is present in the axolemma of the neurohaemal axon terminals and is particularly active during the active state (Roubos et al., 1981a). cAMP-analogues and IBMX can induce the discharge, indicating that an intracellular rise of cAMP is responsible for the start of the discharge *in vivo* (Buma et al., 1986). CDCH-release is calcium-dependent (Roubos et al., 1981b). Furthermore, the cGMP-analogue 8-bromoGMP induces exocytotic release of secretory granule contents (Roubos et al., 1987b). In addition, the intra-axonal pH and inositoltriphosphate seem to play roles in the

induction of the discharge (Moed et al., 1987). Possibly, firstly the auto-excitatory peptide and then CDCH is released. This non-simultaneous release would correlate with the consecutive release of the contents of two morphologically different types of secretory granule (Roubos et al., 1987b).

High CDCH-release during the discharge, demonstrated by bioassay of the haemolymph (Geraerts et al., 1984), is reflected at the ultrastructural level by the frequent occurrence of exocytotic release of secretory granule contents from the neurohaemal axon terminals (demonstrated with tannic acid methods; e.g., Roubos and van der Wal-Divendal, 1980). The exocytozing contents are immunopositive with anti-CDCH (Fig. 5a). Exocytosis is concomitant with a depletion of secretory granules from the terminals (Buma et al., 1984; Roubos, 1984; Roubos et al., 1987a). About 3 hours after the start of the discharge the CDCH titer has decreased to zero (Geraerts et al., 1984). In contrast, the number of exocytosis profiles remains fairly high up to several hours after the end of the discharge. This indicates that the CDC release non-ovulation inducing secretory material during the inhibited state (when the cells are electrically inactive) (Roubos, 1984). Probably, exocytosis during the inhibited state depends on the action of calcium released from mitochondria (which take up excess calcium during the discharge; Buma and Roubos, 1983). It may be suggested that the released material (other CDC peptides?) plays a role in the control of the activity of some components of egg-laying behaviour and/or in the regulation of the secretory activity of accessory sex glands.

Recent immunoelectron microscopy studies indicate that not only CDCH, but also the CDCH-like peptide, αCDCP and calfluxin are released into the haemolymph. This means that the CDC release products into the haemolymph that are derived from both the CDCH- and the CDCH-like precursors. Studies on the relations between the release of these peptides, the electrical state of the CDC and the physiological effects of these peptides are in progress.

THE COLLATERAL SYSTEM - NONSYNAPTIC COMMUNICATION

The cerebral commissure consists of two compartments, separated by a sheath of glial cells. The outer compartment is formed by the neurohaemal area of the CDC, the crossing axons of the ventral CDC run in the inner compartment. These axons branch into blindly ending collaterals, which form an extensive network ("collateral system") throughout the inner compartment. The collaterals never form synaptic contacts; exocytotic release of the contents of secretory granules takes place at nonsynaptic release sites, which lack the characteristic morphology of classical synapses (Schmidt and Roubos, 1987a). Preliminary immunoelectron microscopy studies suggest that not only CDCH (Fig. 5b) but also other CDC peptides (αCDCP, the CDCH-like peptide, calfluxin) are released from the collaterals into the interneuronal space.

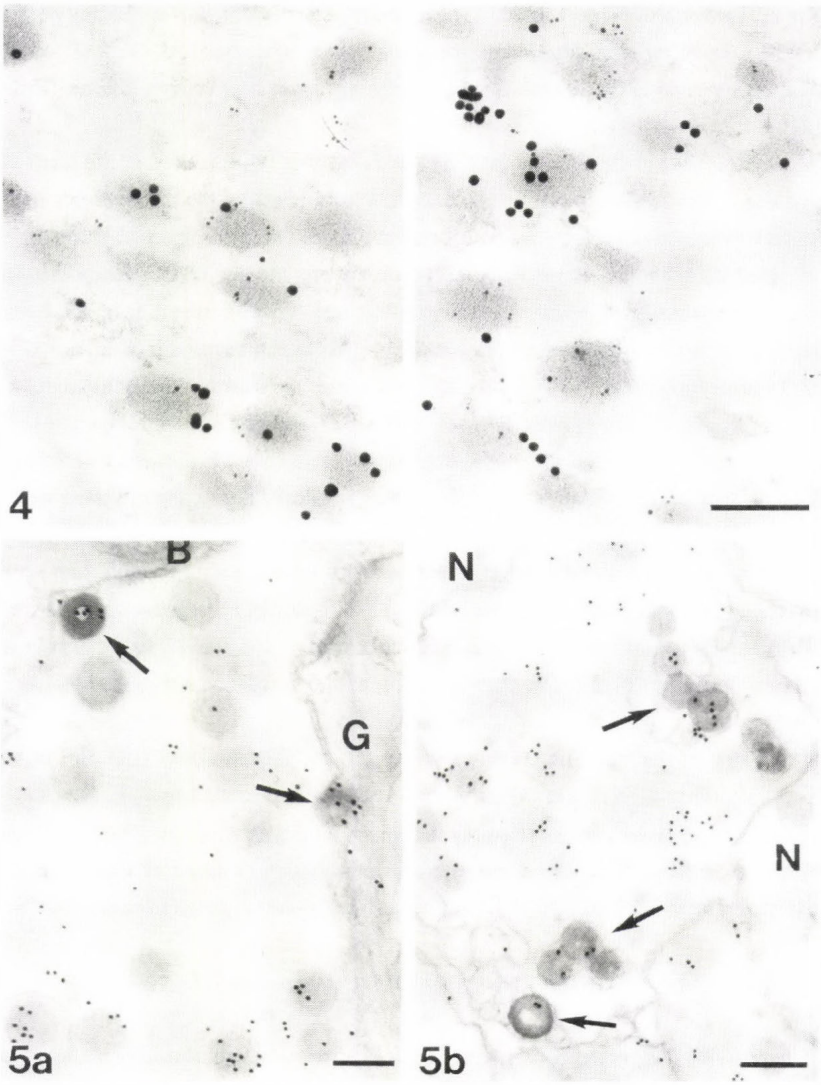

Fig. 4. Immunoelectron microscopy (gold technique, double labelling) with anti-CDCH (small gold particles) and anti-calfluxin (large gold particles). Some secretory granules show colocalization of the respective peptides. Fixation with 0.5% glutaraldehyde. Bar: 0.2 μm.

Fig. 5. Immunoelectron microscopy (gold technique) with anti-CDCH. Neurohaemal axon terminals (a) and collaterals (b) of CDC showing (multiple) exocytosis of immunopositive contents of secretory granules (arrows). Fixation with 0.5% glutaraldehyde and 1% OsO_4. B basal lamina, G glial cell process, N unidentified (non-CDC) neural element. Bar: 0.2 μm.

Using the tannic acid-Ringer incubation-method (TARI-method; Buma et al., 1984) for the detection of exocytotic release of secretory granule contents *in vitro*, it has been found that elevation of the extracellular potassium concentration strongly stimulates exocytosis activity in the collaterals. No stimulation can be induced in the absence of extracellular calcium ions. Electron-dense material occurs apposed at the cytoplasmic side of the axolemma of the collaterals (ethanolic phosphotungstic acid method). This material is homologous with the presynaptic dense projections of the "vesicular grid" in classical synapses. This grid is also present in the CDC neurohaemal axon terminals. Apparently, secretion from nonsynaptic release sites in CDC collaterals shares fundamental characteristics with release from neurohaemal axon terminals and classical synapses: exocytosis, association with a vesicular grid, induction by membrane depolarization, and dependence on extracellular calcium ions (Schmidt and Roubos, 1987b).

It has been proposed that nonsynaptic release of neuronal messengers is involved in nonsynaptic ("diffuse", "paracrine", "at a distance", "hormonal-like") communication within the central nervous system (Roubos et al., 1983; Schmitt, 1984; Schmidt and Roubos, 1987a,b). We suppose that the products released from the nonsynaptic release sites of the collateral system are involved in nonsynaptic communication of the CDC with remote targets in the central nervous system, thus controlling (some components of the) stereotyped phases of egg-laying behaviour. One possible target is the cerebral Ring neuron, which sends an axon branch through the inner compartment and, as was previously shown neurophysiologically, is controlled by the CDC in a nonsynaptic fashion. The Ring neuron controls pedal motoneurons involved in locomotion. In addition, the CDC possibly control buccal and pedal motoneurons nonsynaptically (Jansen, 1984; Jansen and ter Maat, 1985).

In contrast to the neurohaemal axon terminals, the collaterals show maximum exocytosis activity during the resting and inhibited state and low exocytosis activity during the discharge (Schmidt and Roubos, 1987b). This suggests that neurohaemal and collateral release are controlled independently. The glial sheath that separates the inner compartment from the outer may serve as a selective barrier preventing some substances (CDC peptides?) from passing between the two compartments (Schmidt and Roubos, 1987a,b).

Recent light and electron microscope studies show that the collateral system develops almost simultaneously with the neurohaemal axon terminals. It first appears in juvenile snails of 10 mm shell height. During development the collaterals strongly increase in size and, especially, in number. The diameter of the secretory granules increases, reaching a final size in sexually mature snails ($\geq$ 25 mm). Release by exocytosis of secretory material into the intercellular space of the cerebral commissure was observed in collaterals from 10 mm onwards. Immunoelectron microscopy shows that the released material in juveniles is CDCH (-like).

INPUTS TO THE CDC

Various environmental factors, such as food quantity, photoperiod and water temperature, influence reproduction of *L. stagnalis* (Dogterom et al., 1985). Laboratory studies (for refs see Geraerts et al., 1987) have confirmed that these factors influence the rate of oviposition, probably by modulating biosynthesis and/or release of CDCH. Furthermore, intrinsic (neuronal or neurohormonal) factors from the lateral lobes, small ganglionic appendices of the cerebral ganglia, stimulate the rate of oviposition (Geraerts, 1976). Ultrastructural morphometry has demonstrated that the lobes stimulate protein synthesis as well as release activity of the CDC (Roubos et al., 1980). Finally, parasitic infection, tactile stimulation, copulation and clean water stimulation are known to affect CDC activity (for refs. see Geraerts et al., 1987).

The pathways, neural elements and chemical messengers that relay external stimuli to the CDC are only partly known (for reviews see Roubos, 1984; Geraerts et al., 1987). The clean water stimulus, which elicits the CDC discharge, is transferred via the n. analis and n. pallialis dexter externus, bilaterally to the CDC. The socalled SWAP-neuron forms an extensive network in the pedal ganglia and innervates the CDC as well as neurons controlling eating and copulation behaviour. Probably, under normal (non-egg laying) conditions, the network inhibits CDC activity. Tactile stimuli run via all peripheral nerves and project to the CDC via cholinergic synapses on the CDC. Copulation accelerates the onset of maturation of the female reproductive apparatus and of egg-laying, probably via the transfer of substances released from male accessory sex glands. The sensory cells involved may be located in the penis and the vagina.

Recent light immunocytochemical studies and *in situ* hybridization experiments with a cDNA probe encoding CDCH have indicated that CDCH is not only produced by the CDC but also by other neurons in the CNS of *L. stagnalis* (van Minnen et al., 1987). These ectopic CDC may be involved in the control of egg laying and egg-laying behaviour as well. Furthermore, a group of CDC-like cells occurs laterally in each cerebral ganglion. They are much smaller than the CDC and their axons seem to make synaptic contacts with the CDC axons in the cerebral neuropiles. It has been suggested that they control CDC activity in cooperation with similar neurons in the accessory sex glands (see below).

CDC(-LIKE) CELLS OUTSIDE THE CNS

With *in situ* hybridization, Northern blotting and immunocytochemistry it has been shown that CDCH(-like) transcripts and peptides occur in various neural and non-neural tissues outside the CNS (van Minnen and Vreugdenhil 1987; van Minnen et al., 1987; E. Vreugdenhil, unpublished results). Peripheral neurons producing CDCH(-like) material are

present in the oothecal gland, the muciparous gland and the pars contorta, which are female accessory sex glands. In these glands the processes of the neurons terminate on the secretory cells, suggesting that they control glandular release activities. CDCH-immunoreactive material has also been found in secretory cells of the prostate gland and spermduct, as well as in the lumen of the male duct, suggesting that CDCH(-like) peptides are released and transported to the partner during copulation. The biological role of these peptides and their molecular structure are as yet unclear. Possibly, they are derived from different precursors that are encoded by a family of CDCH-genes. The possibility has been raised that all cells expressing one or more of these genes, within as well as outside the CNS, act together in reproduction (van Minnen et al., 1987).

ACKNOWLEDGEMENTS

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DISCUSSION

ARVANOV, V.: There are some data in the literature which explain the bursting activity of some identified nerve cells in molluscs by peptide release. Does the peptide you studied induce bursting type activity of the neurons or not?

ROUBOS, E.W.: The auto-excitatory peptide of the CDC acts specifically upon the CDC, inducing the discharge. This is a prolonged period of beating activity, but bursts may occur, especially when the discharge is evoked in vitro, by adding cAMP analogues or IBMX.

ERDÉLYI, L.: Could you tell us something about the mechanism of the stimuli recognitions which results in activation of the neurosecretion in the cell?

ROUBOS, E.W.: Most likely, the clean water stimulus reaches the CNC via receptors (e.g. for oxygen) in the skin, and a pathway running via the visceral nerves. The receptors on the CDC are not known. Possibly, they are coupled to an adenylate cyclase-cAMP system which is known to induce the CDC discharge.

LEAKE, L.: What is the behaviour of the CDC cells at times of the year when egg-laying is not occurring?

ROUBOS, E.W.: In the laboratory egg-laying occurs throughout the whole year. No systematical studies have been carried out with snails taken in winter from the field, but occasional experiments indicate that their CNC can be brought into discharge by experimental electrical stimulation. So, field conditions may affect biosynthetic secretory events rather than membrane properties. Excitatory messengers have not been identified (ACh inhibits the CDC, FMRF has a modulatory effect).

SYED, N.I.: Could you please tell us which motor neurons are "controlled" by Ring neurone?

ROUBOS, E.W.: These pedal neurons have been studied neurophysiologically but have not been identified topologically and morphologically up to now.

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NEURONES CONTAINING FMRFamide-LIKE PEPTIDES IN THE MODEL INVERTEBRATE SYSTEM, LYMNAEA

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INTRODUCTION

Our objective is to develop defined neuronal systems which utilise peptides as neurotransmitters and/or modulators for molecular, biochemical and pharmacological analysis. We wish to understand the role of these peptides in circuits of known biological function. An essential first step in such long term studies is to discover identified neurones which contain neuropeptides of known structure. In this chapter we will report on the discovery of a number of identified neurones in the snail *Lymnaea* which contain peptides related to FMRFamide. Two of these neurones are excitatory motoneurones of the heart and the third is a centrally acting interneurone. We thus have the opportunity to study both the central and peripheral actions of specific neurones containing this important group of molluscan neuropeptides.

FMRFamide-RELATED PEPTIDES IN *LYMNAEA*

FMRFamide (Phe-Met-Arg-Phe-NH_2) was first detected as a cardioexcitatory agent in bivalve ganglia and later sequenced by Price & Greenberg (1977) in their now famous study. This structure entity is now known to be widespread in various gastropod species but other types of FMRFamide-related peptide have since been discovered which may be more limited in their species distribution (Price, 1987). In *Lymnaea*, at least three distinct peptides have been isolated from

central ganglia and sequenced, FMRFamide itself and two heptapeptides SDPFLRFamide (Ser-Asp-Pro-Phe-Leu-Arg-Phe-NH_2) and GDPFLRFamide (Gly...etc). Non-amidated forms of these heptapeptides were also found (Ebberink *et al.*, 1987).

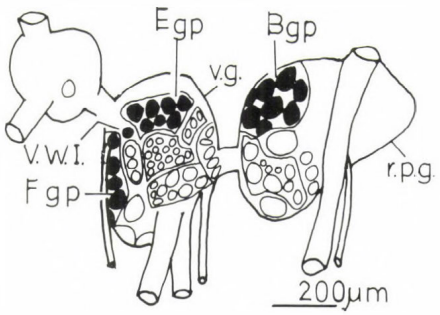

Fig 1. Map of the cell body locations of neurones containing FMRFamide-like peptides in the central nervous system of *Lymnaea*. The Bgp (group) are a cluster of neurones located in the right parietal ganglion (r.p.g.), whilst two other clusters of peptidergic cells, the Fgp and Egp, occur in the visceral ganglion (v.g.). Also within the visceral ganglion is a single identified cell, the visceral white interneurone (V.W.I.), which again contains FMRFamide-like peptides.

The first data concerning the localisation of FMRFamide-like peptides in *Lymnaea* were from the immunocytochemical study of Schot & Boer (1982) who showed cell bodies and processes containing immunoreactive material in all the central ganglia as well as positively-stained fibres in a number of peripheral organs (heart, gut and reproductive system). Three clusters of cells were particularly prominent in Schot & Boer's study and these occurred in the visceral and right parietal ganglia. These clusters appeared to correspond to

the cells described in electrophysiological studies as the Bgp (group) and Fgp (Benjamin & Winlow, 1981) and a more recently discovered group of cells called the Egp (Fig. 1). Subsequent work to be reviewed here showed that cells from all three clusters, as well as a single identified cell located near the Egp, called the visceral white interneurone (V.W.I.) by Benjamin (1984), all contain peptides related to FMRFamide.

IDENTIFICATION OF PEPTIDE-CONTAINING NEURONES

Two main techniques have been used to identify neurones containing FMRFamide-like peptides in *Lymnaea*. 1) Cells iontophoretically filled with Procion Yellow via intracellular microelectrodes were

Table 1

Single-cell RIA (FMRFamide-like peptide/fmol per cell)

Cell-type	Fgp	Bgp	E_{he}	Egp	V.W.I.	Control
No.						
1	390	450	360	510	390	180
2	240	240	240	420	390	<180
3	270	420	480	510	420	<180
4	210	240	690	600	390	<180
5	300	210	180	1020	570	180
6	300	360	210	420	210	<180
7	330	330	330	420	390	<180
8	270	450	240	300	240	210
9	330	<180	270	540	390	<180
10	240	390	<180	180	360	210
x	288	318	310	492	375	132
sd	53	119	171	222	98	55

Student's t-test	Fgp	Bgp	E_{he}	Egp	V.W.I.	Control
Fgp	-	ns	ns	ns	ns	***
Bgp	ns	-	ns	ns	ns	***
E_{he}	ns	ns	-	ns	ns	**
Egp	ns	ns	ns	-	ns	***
VWI	ns	ns	ns	ns	-	***
Control	***	***	**	***	***	-

** = P<0.01 *** = P<0.001 (ns = not significant at 1% level)

immunocytochemically stained by van Minnen & Boer using a polyclonal antibody raised to FMRFamide (Boer *et al.*, 1980). 2) Single cells were dissected from the *Lymnaea* C.N.S. and their contents analysed using a FMRFamide-based radioimmunoassay (Dockray, 1985). Bgp, Egp and Fgp and the single identified neurone, the V.W.I., have all been analysed using these two techniques and shown to contain FMRFamide-like peptides. The exact structural form of the peptide(s) is not clear as both the polyclonal antibodies used in the study are likely to cross-react with the three main FMRFamide-like peptides in *Lymnaea*. The results of the R.I.A. carried out on single dissected cells are shown in Table 1. They indicate that the cells contain considerable amounts of immunoreactive FMRFamide-like peptides, significantly more than controls taken from the H, I, J, K clusters (cells described in Benjamin & Winlow, 1981). On average each of the cell types contained between 300 and 500 fmole of immunoreactive peptide with no significant differences between the different cell types in the levels of peptides present.

E_{he} CELLS: CARDIOACTIVE FMRFamide-CONTAINING MOTONEURONES

FMRFamide is classically a cardioexcitatory substance (e.g. Price & Greenberg, 1980) and it seemed likely that some of the FMRFamide-containing neurones in the parietal and visceral ganglia of *Lymnaea* would be heart motoneurones. In order to test this hypothesis individual neurones in the Egp, Fgp and Bgp were activated by intracellular current injection and the effects on heart beat tested. The preparation consisted of isolated heart and associated organs like the kidney connected to the isolated brain by the intestinal nerve. The heart and CNS could be independently perfused by salines of various ionic compositions or with added drugs. Mechanical recordings were made from the heart to monitor overall changes in heart output or alternatively intracellular recordings from single auricular muscle fibres allowed more detailed examination of the electrical responses to motoneuronal stimulation. Finally, the axonal projections of neurones were examined in whole mounts following Lucifer Yellow injection (methods in Elliott & Benjamin, 1985).

Only two cells had consistent effects on heart beat. These were located in the Egp and have been called the E_{he} (heart excitor) cells. These cells were normally silent in the isolated C.N.S. but a burst of spikes increased the beat rate of the heart (Fig. 2). Other cells in the Egp or other FMRFamide-containing cells had no effect on heart

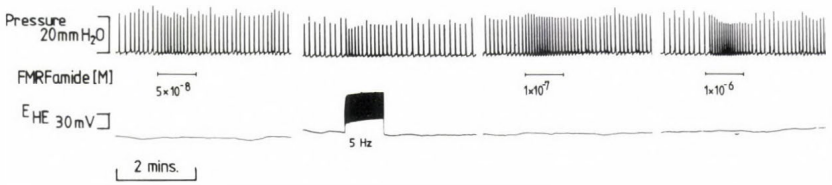

Fig. 2 An evoked burst of spikes in an E_{HE} cell (heart FMRFamide-containing motoneurone) accelerates heart beat. Similar responses are produced by perfusing FMRFamide through the heart.

beat. The special status of the E_{he} cells in relation to other Egp cells was indicated by two further results; they were the only cells to be consistently electrotonically coupled and they alone had a single main axon projecting along the intestinal nerve, the main nerve innervating the heart. Other neurones within the Egp had much more widespread projections to other parts of the brain and to many other peripherally projecting nerves including the intestinal nerve. This suggested a much wider distribution of peptide release for the Egp cells in general compared with the E_{he} cells. It has not been possible to follow the axons of the E_{he} cells right into the heart muscle tissue but we know that they at least reach the branch of the nerve close to the junction of the anterior aorta with the pericardium. They thus have the appropriate projections for heart motoneuronal function and further evidence for direct effects on the heart comes from intracellular recordings from heart muscle cells (Fig. 3). Here activation of the E_{he} cells led to e.j.p.s (excitatory junctional potentials) in single muscle fibres. This innervation

from a particular cell appeared to be widespread because recordings from randomly selected auricular muscle fibres always produced e.j.p. responses following a burst of spikes in the E_{he} cell (Fig. 3). In separate experiments mechanical recordings showed that the ventricle was also innervated by the E_{he} cells. Recordings shown in Fig. 3 showed that the responses were always compound e.j.p.s but in other preparations small unitary e.j.p.s (<0.5mV in amplitude) could be

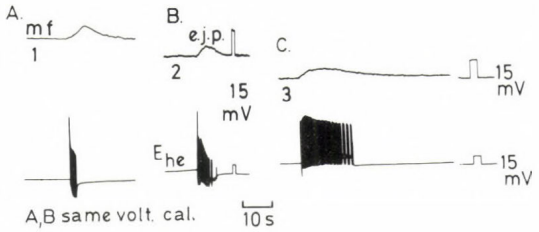

Fig. 3 E.j.p.s evoked in heart muscle fibres by bursts of spikes in a E_{he} cell (heart FMRFamide-containing motoneurone) accelerates heart beat. Similar responses are produced by perfusing FMRFamide through the heart.

recorded, following each E_{he} spike 1:1 with constant latency. Action potential-like responses could always be triggered if strong bursts of spikes in E_{he} cell were evoked. On the basis of the present anatomical and electrophysiological evidence it seems likely that the E_{he} cells are motoneurones having direct effects on the heart. Any indirect effects via other central neurones are ruled out because blocking of central chemical synapses by a special cobalt saline does not prevent the E_{he} cells exciting the heart.

Can we prove that the cardioexcitatory effects of E_{he} cells are due to release of FMRFamide-like peptides? One problem is that we do not have an antagonist for this family of peptides. Another is that

further cardioactive substances might be co-localised with FMRFamide and be mainly responsible for the motoneuronal effects. Evidence that FMRFamide is the main substance released from the E_{he} cells came from experiments in which the effect of motoneuronal stimulation was compared with direct application of peptides to the heart. In Fig. 2 application of synthetic FMRFamide between 10^{-6} and 10^{-7}M produced very similar responses to direct motoneuronal stimulation. Application of other candidate cardioregulatory peptides such as substance P or SCP_A and $_B$ either resulted in much slower more complex effects (SCP) or required very high threshold concentrations to produce a response (substance P). Classical neurotransmitters such as 5-HT and dopamine also have cardioacceleratory effects on the *Lymnaea* heart at low concentrations but blocking their actions with antagonists did not prevent the E_{he} cells from exciting the heart. All the evidence so far, although indirect, supported the hypothesis that the E_{he} cells release FMRFamide or a related peptide.

THE VISCERAL WHITE INTERNEURONE (V.W.I.)

This is a single identifiable neurone first described as having postsynaptic effects on neurosecretory neurones in the *Lymnaea* C.N.S. (Benjamin *et al.*, 1983; Benjamin, 1984), although synaptic responses in a much wider population of neurones are now known (Benjamin *et al.*, 1985). The V.W.I. has an interesting ring-like anatomy with processes confined to the C.N.S. (Benjamin, 1984), so it appears to be a true interneurone. It was reported above that the V.W.I. contains FMRFamide-like peptides and it is tempting to assume that the synaptic responses illustrated in Fig. 4 might be mediated by members of this peptide family although again the role of co-transmitters cannot be ruled out.

Postsynaptic responses were inhibitory (Fig. 4A), excitatory (Fig. 4B) or biphasic (Fig. 4C). Mostly, the responses were weak so that a burst of spikes in the V.W.I. were required to see a postsynaptic potential. We are currently examining the details of these synaptic events and some of these results are illustrated in Fig. 4. For

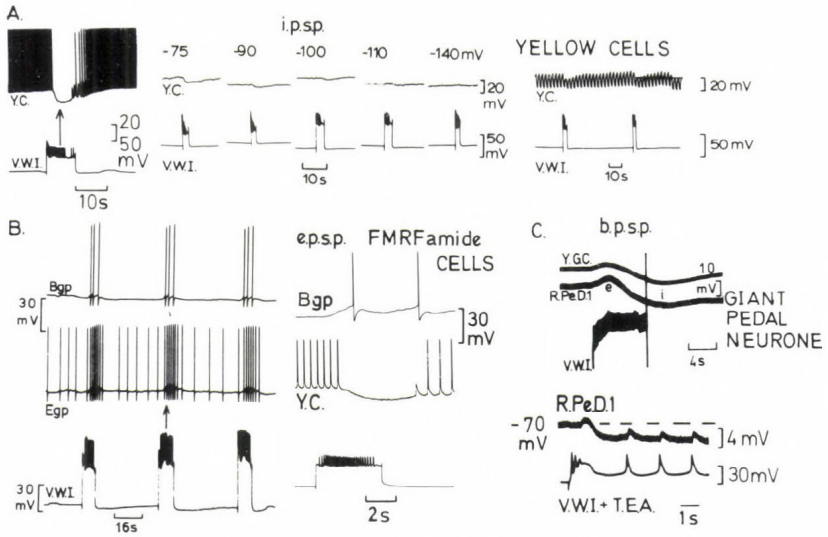

Fig.4 The FMRFamide-containing neurone, V.W.I. (visceral white interneurone), produces a variety of postsynaptic responses in *Lymnaea* CNS neurones. A. Inhibitory responses (i.p.s.p.s) in the Yellow Cells (Y.C.). Left hand panel shows that a burst of spikes in the V.W.I. leads to cessation of firing in the Y.C. Middle panel shows that progressively manipulating the membrane potential of a Y.C. in a hyperpolarising direction eventually leads to the disappearance of the i.p.s.p. with an apparent equilibrium potential of between -110 and -140 mV. Right hand panel shows that the i.p.s.p. in the Y.C. is due to a conductance increase mechanism. B. Excitatory responses in Bgp and Egp cells (left hand panel). Right hand panel shows an excitatory response in a Bgp cell accompanied by an inhibitory response in a Y.C. C. Biphasic synaptic responses in a yellow-green cell (Y.G.C.) and giant pedal neurone (R.Pe.D.1). The b.p.s.p. consists of an initial depolarising wave (e) followed by a longer-lasting hyperpolarising potential (i) (top panel). The bottom panel shows that spikes in the V.W.I. can produce 1:1 e.p.s.p.s in R.Pe.D.1. These are superimposed on a summated i.p.s.p. which maintains a constant hyperpolarisation from the initial membrane potential of -70mV. The duration of spikes in the V.W.I. was enhanced by injecting T.E.A. into the neurone via the recording micropipette.

instance the inhibitory response seen in the Yellow Cells involved a conductance increase mechanism with a reversal potential of more than -100 mV (Fig. 4A). In general the responses are very delayed (it often takes several seconds to see the start of the response) and it often takes many seconds to reach maximum amplitude. The 'slowness' of the response usually makes it difficult to see unitary responses. An exception to this is the initial depolarising response in the pedal ganglion giant dopamine-containing neurone (R.Pe.D.1) where enhancing the duration of the V.W.I. spike using TEA gives clear unitary e.p.s.p.s following 1:1 from each spike (Fig. 4C). Of particular interest from the point of view of the present chapter are the slow excitatory responses in the Bgp and Egp cells (Fig. 4B). If indeed the V.W.I. responses are due to the effects of peptide release then we appear to have a hierarchy of control amongst the FMRFamide-containing cells with one type of cell capable of exciting another cell containing similar types of peptides.

CONCLUSIONS

We have identified a number of neurones within the *Lymnaea* C.N.S. which contain FMRFamide-like peptides. They occur in three main clusters in the visceral and right parietal ganglia. Within one of the visceral ganglion clusters (the E gp) two special neurones, the E_{he} cells, were found which had distinct anatomical and physiological characteristics. Good experimental evidence suggested that they were excitatory motoneurones acting on the heart. We argued that their main effects could be mediated by FMRFamide-like peptides. Further work is now in progress to determine more precisely the structural form of the peptide present in the E_{he} cells. It is also necessary to show that the peptide(s) are released by the E_{he} terminals in the heart. We need to know if the E_{he} cells contain other bioactive substances which may play an important role in heart excitation. The same question arises for the V.W.I., a centrally-acting interneurone also shown to contain FMRFamide-like peptides in the present study. Its effects may be at least partly mediated by co-released substances, although FMRFamide-related peptides are still the prime candidate transmitter(s). The V.W.I. is an example of an interneurone with multiple postsynaptic actions in the C.N.S. Whether its effects are

monosynaptic is not yet clear except in the case of the e.p.s.p.s recorded in the pedal dopamine-containing giant neurone where unitary responses were clear.

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DISCUSSION

ELEKES, K.: Did you apply FMRFamide-immunocytochemistry on the heart muscle?

BENJAMIN, P.R.: This has been done by Schott and Boer and they stained nerve fibres in both auricle and ventricle using a polyclonal antibody.

FLOREY, E.: 1. Do you have any evidence that what you call heart motoneurons make synaptic contacts with the heart muscle cells?

2. You are not actually suggesting that in Lymnaea the origin of the heart beat is neurogenic?

BENJAMIN, P.R.: 1. Not directly, although FMRFamide containing nerve fibres were described in the heart by Schott and Boer. Also nerve terminals containing large-core vesicles were also found in electron microscopy. Whether there are classical synapses is still unknown.

2. Isolated hearts will beat as long as some pressure is applied, so they are basically myogenic. However, they do receive complex neural innervation of several types.

JANSE, C.: 1. Are there, apart from the E_{He} neurons, more neurons involved in heart control and where are these located?

2. How did you identify the E_{He} neurons for the biochemical studies?

BENJAMIN, P.R.: 1. Yes, there are 4 more types of excitatory motoneurons and one inhibibory type. All are in the visceral ganglion.

2. We first stimulated them electrically to see if they excited the heart, then dissected them.

KITS, K.S.: Does the response to FMRFamide desensitize and if so, can you block the e.j.p. by application of FMRFamide to the bath?

BENJAMIN, P.R.: We have seen no clear evidence for desensitization.

SYED, N.I.: I am excited to see that the visceral E-group cells are heart motoneurons because these cells, in our preparations, receive excitatory locomotor inputs. Furthermore, they also receive excitation from a pair of locomotor interneurons which we have recently identified.

BENJAMIN, P.R.: You have to be careful. Only two of the E group cells are heart motoneurons and there are many other non-cardioactive cells in the same group. In general, your results sound very interesting as one would predict that heart rate and cardiac output would be regulated in relation to behaviour such as locomotion.

WINLOW, W.: From the diagrams we have seen today I am almost certain that the visceral E group cells are neurons receiving inputs from a large pair of locomotor interneurons in the pedal ganglia (L./R. Pe. D.11) as Mr. Syed has suggested. Clearly there must be integration between cardiac output and locomotor activity.

BENJAMIN, P.R.: I agree that there must be integration. I only wanted to make it clear to Syed that only a small proportion of the Egp cells are directly involved with heart function and that to identify E_{He} cells you have to test their effects on the heart.

WALTHER, C.: How good is the evidence that in your animal FMRFamide exists rather than FLRFamide - since your extended peptides do contain leucine instead of methionine?

BENJAMIN, P.R.: FMRFamide has been sequenced from the Lymnaea CNS. There may be FLRFamide but it probably does not occur in large quantities.

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THE DUAL INHIBITORY ACTION OF FMRFamide ON CAUDO DORSAL CELLS IN THE POND SNAIL INVOLVES A SINGLE RECOGNITION SITE

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SUMMARY

Research in our laboratory has shown that FMRFa-like immunoreactivity occurs in varicosities closely associated with the Caudo Dorsal Cells (CDCs) in *Lymnaea stagnalis*. Electrophysiological studies revealed that FMRFa effectively inhibits the neuronal activity of the CDCs via a dual membrane action at low concentrations. This dual action consists of two independent parts, being (i) the induction of a transient potassium dependent hyperpolarizing response (blocked by 4-AP) and (ii) the inhibition of the excitability of these cells (not blocked by 4-AP). To clarify the identity of the recognition site(s) involved, we have focused attention on the structure-activity relations of FMRFa in this system. We have screened the effects of all sorts of synthetic FMRFa analogs, as well as naturally occurring FMRFa-related heptapeptides. This revealed that the structural-activity relationships of both responses are identical and that while the first two amino acids of FMRFa can be substituted by related residues without any loss of bioactivity, the Arg^3-Phe^4-NH_2 sequence is essential. GDPFLRFa and SDPFLRFa were equi-active to FMRFa. In addition we have screened the effects of the alpha Bag Cell Peptides (alphaBCPs) from *Aplysia* , and found that these structures mimic the FMRFa effects on CDCs to a large extent. The consequences of the structure-activity analysis of FMRFa are discussed and it is concluded that a single type of recognition site on CDCs mediates both types of responses to FMRFa-like peptides.

INTRODUCTION

Background

The Caudo Dorsal Cells (CDCs), which control egg laying in *Lymnaea stagnalis*, release several peptides including the egg-laying hormone, during a 45-60 minute firing period, the afterdischarge (Geraerts & Bohlken, 1976; Kits, 1980; Ebberink et al., 1985; Maat et al., 1986). *In vitro* this afterdischarge can only be evoked in excitable, so called resting state CDCs and it is initiated by cAMP-dependent activation of the endogenous pacemaking

mechanism of the cells (Kits, 1980; Kits & Bos, 1981; Roubos et al., 1981; Geraerts et al., 1983; Geraerts & Hogenes, 1985; Buma et al., 1986). Resting CDCs respond to short intracellular electrical stimulation of one cell in a highly characteristic manner by producing a Depolarizing After Potential (DAP). This DAP is distributed throughout the network of the 100 CDCs, probably by means of chemical transmission (Maat et al., 1986). FMRFa-like immunoreactivity is widely distributed in the CNS of *Lymnaea* (Schot & Boer,1982). Biochemical analysis has shown that about 20% of this immunoreactivity is due to FMRFa and 80% results from SDPFLRFa and GDPFLRFa (Ebberink et al., 1987). Of special interest is the presence of antiFMRFa positive varicosities, which occur closely associated with the CDCs (Brussaard et al., in press).

The dual action of FMRFa

FMRFa has a dual inhibitory action on the CDCs: (i) a transient hyperpolarizing response, which desensitizes (H-response) and (ii) an inhibition of the excitability, which is measured as a suppression of the DAP and lasts as long as the peptide is present in the bath. We have found that the two responses of CDCs to FMRFa are independent and that both responses occur in a dose dependent and reversible manner (Brussaard et al., in press). Furthermore both effects of FMRFa occur in silent resting state CDCs as well as in discharging cells. This holds true for CDC-recordings from intact CNS preparations using bath applications of the peptide but also for isolated cells using pressure ejection methods. While the occurrence of antiFMRFa positive varicosities closely associated with the individual CDC somata and axons were indicative of a physiological relevance of the *in vitro* effects of FMRFa on CDCs, the experiments with isolated cells showed clearly that CDCs do possess receptors to FMRFa-like peptides. The H-response is not blocked by any of the known antagonists to classical neurotransmitters and K^+ has shown to be the major charge carrier in this response. The latter conclusion was substantiated in experiments in which the reversal potential of the H-response showed to be solely dependent on the external K^+ concentration. Evidence for the independence of both parts of the dual action of FMRFa came from experiments which showed that the H-response is blocked by 4-Aminopyridine (4-AP), whereas the suppression of the DAP is not (Fig. 1). The present study addresses the question of whether this dual action is mediated through a single type of recognition site or not.

QUANTITATIVE ANALYSIS OF THE PEPTIDE RECEPTOR INTERACTION

In our studies we obtained parallel log dose response curves for the H-response and the amount of DAP-suppression to FMRFa. CDC recordings from intact CNS preparations revealed that maximal H-responses are induced with doses of ≥ 50 μM FMRFa, the ED_{50} being 2 μM. Although CDCs fail to generate afterdischarges at FMRFa concentrations as low

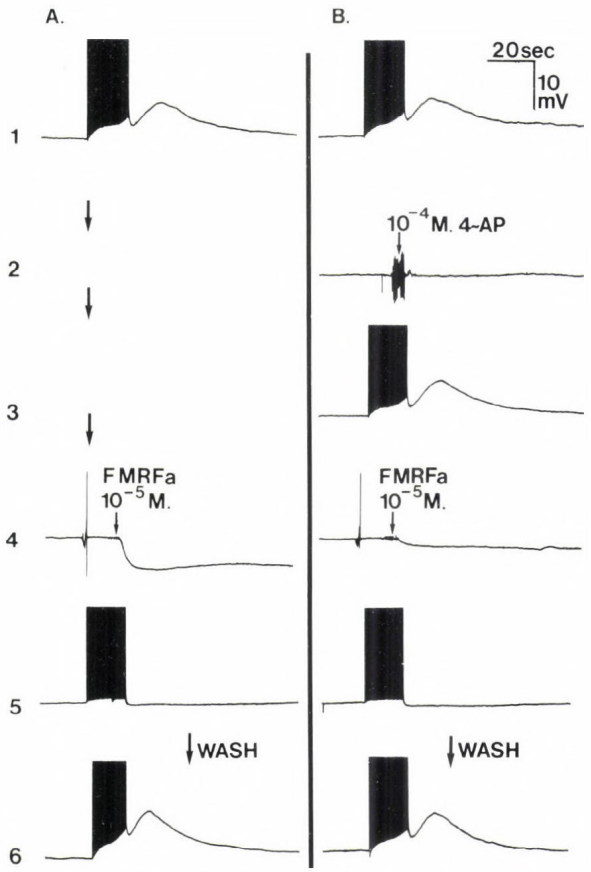

Fig. 1: The effect of 4-AP incubation on the occurrence of both FMRFa responses at resting membrane potential in recordings from CDCs in intact CNS. (A) Recording of a control experiment, testing the effects of FMRFa on the membrane potential (A4) and the excitability of the cell (A5). Steps 1 to 4 are a continuous recording. (B) Using the same preparation the effects of FMRFa were tested in the presence of 4-AP. Application of 4-AP alone did not affect the DAP produced by electrical stimulation of the cell (B3), indicating that it did not itself affect excitability. Subsequent application of FMRFa produced hardly any H-response indicating that 4-AP blocked the channels involved in the H-response(B4). FMRFa still suppressed the DAP (B5), whereas a wash restored the original conditions of the cell (B6) (n=5) (Action potential top not shown).

as 0.01 μM, 100% suppression of the DAP only occurs at about 1 μM, with an ED_{50} of 0.007 μM. From these results no conclusions can be drawn about the affinity characteristics of the receptors involved, since the difference in sensitivity of both CDC responses can also be ascribed to other parts of the mechanism like different intracellular amplification steps through second messengers. However when the same type of quantitative analysis of FMRFa effects was conducted using isolated CDCs in primary culture, the H-response curve was shifted to lower concentrations (ED_{50} = 0.01 μM), whereas at concentrations of ≥ 0.01 μM

FMRFa the cells failed to generate discharges. Thus, while the same range of concentrations of the peptide has similar effects on the CDC excitabilitiy in both types of preparations, isolated cells are about 200 times more sensitive to FMRFa with respect to the induction of the H-response. This means that the dose-response relations of the DAP-suppression in the CNS (ED_{50} : 0.007 µM) and the induction of the H-response in isolated cells (ED_{50}: 0.01 µM) do show overlap. We assume therefore that the affinity of the receptors for FMRFa-like peptides and the effectiveness with which FMRFa induces both responses are very similar. Thus, although the electrophysiological characterization of the FMRFa effects clearly indicated that there is a duality in the CDC response, the quantitative analysis of the effects was indicative of a single type of recognition site involved in the mediation of these effects. The observed shift of the threshold for H-responses in the different types of preparation, could be explained by assuming that presynaptic structures on CDCs in the CNS act as a diffusion barrier to extrasynaptically applied FMRFa.

STRUCTURE ACTIVITY ANALYSIS

Further evidence for the 'single recognition site' hypothesis came from a series of experiments in which we screened the effects of synthetic FMRFa analogs (Table 1). Where possible, each amino acid in this tetrapeptide sequence was substituted by a related and an unrelated residue. Furthermore, shorter sequences like NlRFa (Nl = Nle), RFa and non-amidated FMRF were tested. Each analog was screened for both its ability to induce the H-response and the suppression of the DAP. None of these analogs was selective for one part of the dual action and whenever a substance was less effective than FMRFa, the loss of bioactivity was reflected to an equal extent in both responses of the CDCs. Several substances cross-desensitized with FMRFa, hence they were agonistic and none of the substances listed were antagonistic to FMRFa. With respect to the structure activity relationship it is concluded that the C-terminal Arg^3-Phe^4-NH_2 is essential for both CDC-responses to FMRFa. In this respect the structure-activity relations of FMRFa on the CDCs parallel other studies of the bioactivity of FMRFa-like peptides (Price & Greenberg, 1980; Painter et al., 1982). Even slightly modified tetrapeptides with Lys^3 or Trp^4 showed little if any activity. Non-amidated peptides were also ineffective. However in addition to this C-terminal sequence, which on its own is ineffective, at least two more amino acid residues should be linked to Arg^3. This is because the absence of the Phe^1 in NlRFa caused a considerable loss of bioactivity, while FNlRFa was equipotent to FMRFa. Further analysis revealed that amino acid residue at position 1 should have an aromatic and nonpolar R-group. This does not necessarily have to be that of Phe because WNlRFa was about equipotent to FMRFa, but RMRFa was ineffective up to 100 µM. The amino acid residue at position 2 should have a nonpolar R-group like Met, Leu or Nle, but not Gly. Final evidence for the 'single recognition site' hypothesis was found with a new analog:

Dansylated-Arg-Phe-NH_2 (DNS-RFa). This substance mimicked all FMRFa effects on CDCs; it induced DAP suppression (in intact CNS preparations) and H-responses (in isolated CDC preparations), both in a dose dependent manner parallel to the FMRFa curves (not shown), and it arrested ongoing discharges as illustrated in Fig. 2. Although the equimolar ratio of FMRFa and DNS-RFa is 1:100, clear cross-desensitization between FMRFa and DNS-RFa

Table 1. Structure activity relationship

PEPTIDES	H-RESPONSE %		DAP-SUPPR. %	
	5 x 10^{-6}M	10^{-4}M	5 x 10^{-6}M	10^{-4}M
F M R F a	54	100	100	100
F M R F	0	0	0	0
F L R F a	54	100	100	100
F L R F	0	0	0	0
F Nl R F a	54	100	100	100
WNl R F a	44	100	85	100
R M R F a	0	0	0	0
F G R F a	0	38	10	70
F M K F a	0	27	10	100
F M RW a	0	0	0	0
F M R L a	0	0	0	0
Nl R F a	10	32	25	75
R F a	0	0	0	0
R F	0	0	0	0
DNS-R F a	+	++	75	100
DNS-F a			0	0
F a	0	0	0	0
F	0	0	0	0

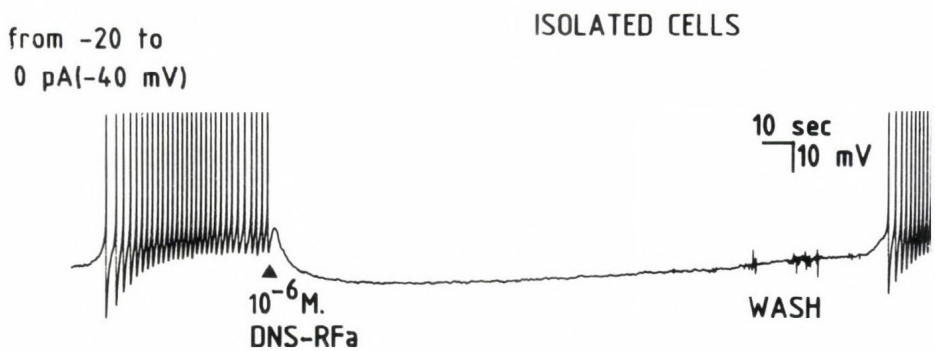

Fig.2: DNS-RFa mimics FMRFa in that it is capable of instantaneously arresting an ongoing discharge in isolated CDCs. The cell in this experiment was discharging upon impalement, and was kept silent, using 20 pA hyperpolarizing current until the start of the experiment. FMRFa is about 100 times more potent in arresting discharges than DNS-RFa. After rinsing the bath the CDC resumed firing again (n=5).

was observed (not shown). Thus the structure activity relations for induction of either of the two CDC responses to FMRFa are identical, hence we conclude that an identical recognition site on the CDCs is involved in both parts of the dual action of FMRFa.

FMRFa RELATED NATURALLY OCCURRING HEPTAPEPTIDES

In this study we also included the effect of changes in chain length on the bioactivity of peptides as summarized in Table 2. In keeping with the ideas of the 'single recognition site' hypothesis and the structural requirements described above, we found here that N-terminal elongation does not affect the bioactivity with respect to either of the two CDC responses. This holds true both for the native *Lymnaea* heptapeptides SDPFLRFa and GDPFLRFa as well as for pQDPFLRFa from *Helix*. Recently, Cottrell & Davies (1987) suggested that the 4 distinctive effects of native tetra- and hepta-peptides on various identified neurons in the CNS of *Helix* are due to the existence of multiple receptor sites, which are specific for either tetra- or hepta-peptides. In our system we found evidence for yet another type of FMRFa receptor in the brain, one which is equally sensitive to both types of native molluscan peptides. In this respect, the receptor on the CDCs of *Lymnaea* resembles the presumably ancestral type of FMRFa receptors found on cardiac and noncardiac muscles in more primitive animals (Price and Greenberg, 1980; Painter et al., 1982; Price, 1986). These findings have several implications on how FMRFa is likely to regulate the neural activity of the CDCs. Price (1986) discussing the evolution of FMRFa related peptides, suggested that FMRFa is the ancestral peptide of its family, which might mean that the CDCs possess an evolutionary ancient type of FMRFa recognition-site for FMRFa-like peptides. He argued that in pulmonate snails, like *Lymnaea* and *Helix*, FMRFa itself serves as a transmitter and that the heptapeptides may serve as neurohormones. It is known that FMRFa-like peptides circulate in the blood of *Lymnaea* with concentrations of upto 10^{-8} M (Price, 1986). This is slightly above the ED_{50}

Table 2. Structure-activity of related peptides

PEPTIDES	H-RESPONSE %		DAP-SUPPR. %	
	5×10^{-6}M	10^{-4}M	5×10^{-6}M	10^{-4}M
Lymnaea				
FMRFa	54	100	100	100
SDPFLRFa	50	100	100	100
GDPFLRFa	52	100	100	100
Helix				
pQDPFLRFa	50	100	100	100
Synthetic				
PFLRFa	54	100	100	100
Aplysia				
APRLRFYSL	50	76	100	100
APRLRFYS	50	76	100	100
APRLRFY	55	82	100	100

for the DAP suppression and at this concentration the CDCs would fail to generate discharges. However it is unlikely that such concentration would affect the membrane potential of the CDCs *in vivo* , since this concentration is about the threshold of the H-response in intact CNS preparations. Moreover the H-response is a desensitizing response. From our immunocytochemical data it is clear, that (*in vivo*) the origin of the FMRFa-like peptides might well be presynaptic neurons which release their products onto the CDCs either synaptically or non synaptically. Thus we have to consider the possibility that CDCs have two pools of receptors to FMRFa-like peptides, one for neurohormones and one for neurotransmitters, however the respective effects will be mediated by the same type of recognition site.

ALPHA-BAG CELL PEPTIDES

Recently, ter Maat et al. (1987) reported that excitatory feedback occurs in the CDC system chemically mediated by small peptides. The question arises whether these peptides might interfere with FMRFa binding to its receptor. In the bag cells system of *Aplysia californica* , which may be regarded as an analog of the CDCs in *Lymnaea* , the alpha Bag Cell Peptides (alphaBCPs) trigger discharges that appear very similar to the afterdischarges evoked by electrical stimulation (Rothman et al., 1983). However in experiments in which the effects of alpha BCPs on the CDCs were screened no excitatory effects were observed. In contrast we found indications that alpha BCPs interact with the FMRFa receptors on CDCs (Table 2). Though overall FMRFa was 10 times more potent, the alpha BCPs both induce typical H-responses and inexcitability of CDCs in both type of preparations used (intact CNS and isolated cells). The alpha BCPs also were capable of arresting ongoing CDC discharges and partially cross-desensitized with FMRFa. The effects were irrespective of whether the C-terminal elongation was YSL (Tyr-Ser-Leu), YS or Y. Thus, in original targets these peptides have excitatory effects, whereas in the CDC system of *Lymnaea* they mimic inhibitory actions of peptides which at first sight seem unrelated. The relevance of this is not clear yet, however the alpha BCPs all have an -Leu-Arg-Phe sequence and to some extent this meets the structural requirements to activate FMRFa receptors on the CDCs. This would imply that the NH_2-group of FMRFa is not essential but can be exchanged by Tyr without severe effects on the bioactivity. We are currently focusing on the identity of the homolog CDC peptides involved in the excitatory feedback, in order to clarify the question of whether a possible interference with the regulatory action of FMRFa occurs at receptor level or not.

CURRENT MODEL

Although FMRFa has a dual inhibitory action on CDCs - inhibiting their neuronal activity by means of two independent ways of regulation - these cells possess only one type of

recognition site according to the structure activity study. Moreover tetra- and heptapeptides of the 'FMRFa' family are equipotent in this system. The immunocytochemical data suggest that *in vivo* the origin of FMRFa-like peptides might well be neurons which release their products onto CDCs either synaptically of nonsynaptically. On the other hand FMRFa-like peptides may circulate in the blood in concentrations up to 0.01 μM. At this concentration the excitability of the CDCs would be affected. Tentatively it is hypothesized here that the transient desensitizing H-response to FMRFa might well reflect the action of a synaptic transmitter since upon isolation of the CDCs the threshold for induction of the H-response was shifted to lower concentrations. Due to the isolation procedure which involves a trypsine treatment, the receptors on isolated cells can be regarded as extrasynaptic. Therefore the finding that the dose response curves of the H-response in isolated cells and the DAP-suppression from intact CNSs do show overlap, indicates that the latter response involves extrasynaptic receptors which are to be activated by neurohormones and/or by nonsynaptically released transmitters. The finding that the suppression of the DAP by FMRFa did not desensitize for tens of minutes, is in keeping with this hypothesis. Further research is necessary to meet all criteria required to identify the involvement of a FMRFa-like synaptic transmitter.

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DISCUSSION

BENJAMIN, P.: I was surprised that the heptapeptides appeared to have the same biological activity as the tetrapeptides in your study. Prof. Cottrell found differences in the effects of these two peptides on Helix neurones.

BRUSSAARD, A.B.: Apparently here we have found a fifth type of FMRFamide receptors in the CNS of molluscs. Now, David Price suggested recently that the tetrapeptide FMRFamide is the ancestral peptide in its family. Later on in the evolution FLRFamide and the heptapeptides evolved. As a result also receptor evolution may have taken place, which means that some targets retained their original type, and others developed new types, either specific for tetrapeptides or for heptapeptides. So probably CDCs remained expressing the original type of receptors.

ERDÉLYI, L.: 1. Have you tested the effects of other potassium channel blockers such as Ba^{2+} or TEA^{+} on the FMRFamide-evoked response?

2. Do you know what the ionic mechanism of the DAP is? Is it caused by a decrease of potassium conductance?

BRUSSAARD, A.B.: 1. We have tested the effect of TEA. It does not affect our potassium dependent H-response to FMRFamide. We observed linear I-V curves, which suggest voltage insensitivity and the reversal potential of the H-response depends solely on the external potassium concentration.

2. We have not clarified that part yet.

KLEE, M.R.: In some Aplysia neurons 4-AP blocks different kinds of ACh-potentials. Especially slow potassium dependent potentials are reduced by 4-AP in the micromolar range (see Klee et al., this Symposium).

BRUSSAARD, A.B.: In CDC's we also have effects of acetylcholine, however, these responses do not interfere with FMRFamide re-

sponses. Moreover various cholinergic antagonists do not cross-desensitize with the FMRFamide induced H-responses in CDC's, so we are probably talking about different responses here.

LUKOWIAK, K.: Have you examined the effect to ARACAclonic acid on these cells? Do you think it will have the same effect as FMRFamide?

BRUSSAARD, A.B.: We have not done that yet, but in theory FMRFamide receptor activation can be coupled to any second messenger system that we are aware of.

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PURIFICATION AND PRIMARY STRUCTURE OF PEPTIDE PRODUCTS OF THE EGG-LAYING HORMONE GENE FAMILY IN APLYSIA

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Egg-laying behavior in the marine opisthobranch mollusc Aplysia is elicited by a neuropeptide known as the egg-laying hormone (ELH). ELH is synthesized in the bag cells, two symmetrical clusters of approximately 400 neurons located in the rostral portion of the abdominal ganglion at the base of the pleurovisceral connectives. Injection of homogenates of the bag cells into sexually mature Aplysia induces egg laying. The bag cells are normally electrically silent, but can be triggered to fire a synchronous burst of action potentials, termed an afterdischarge, lasting up to 30 min or longer (6). In vitro experiments have demonstrated that ELH secretion occurs only during an afterdischarge (25). Moreover, in vivo recordings from intact, freely behaving animals have shown that an afterdischarge, whether spontaneous or induced, is always followed by egg laying, indicating that the activity is behaviorally relevant (16). The egg-laying hormone of Aplysia californica has been chemically characterized and is a 36-residue, basic peptide (4). Molecular genetic studies have demonstrated that ELH is encoded by an ELH gene, one member of a small family of approximately four to five genes per haploid genome (23). These genes are expressed in a tissue-specific manner in the animal, such that the ELH gene is expressed in the bag cells and specific populations of central neurons (2,3,10,14,17,23,24); other genes of the family are expressed in the atrial gland (9,11,22,23), an exocrine organ of unknown function that secretes its products into the oviduct (1,15). The ELH gene predicts the synthesis of a 271-residue precursor polyprotein which is proteolytically processed to liberate ELH and several other peptides, including an acidic peptide (AP), alpha bag cell peptide (BCP), beta BCP, and gamma BCP (4,13,20,23).

The bag cells of Aplysia californica have been extensively studied, yet

significant morphological, electrophysiological, and behavioral insights into the bag-cell system have been obtained from investigations of a related species, Aplysia brasiliana. (e.g. 6,8,16). The neuropeptide egg-laying hormone that induces egg deposition in this species has not been characterized, however. In this report, we describe the chemical characterization of the egg-laying hormone of Aplysia brasiliana, including the complete primary structure. We then describe the chemical characterization of processing products of ELH-family genes that are expressed in the atrial gland of Aplysia californica. The significance of these polypeptide structures is interpreted in light of existing nucleotide sequence data for the ELH-family genes derived from analyses of cDNA and genomic clones.

PRIMARY STRUCTURE OF APLYSIA BRASILIANA ELH

The bag-cell clusters from 152 A. brasiliana were extracted in an acidic solution (1 M acetic acid, 20 mM HCl) containing four peptidase inhibitors (phenylmethylsulfonyl fluoride (PMSF), phenylalanylalanine (Phe-Ala), leupeptin, antipain), and the extract was chromatographed on a Sephadex G-50 superfine column under acidic conditions. The 1.5-10-kDa peptide fraction (Fraction II in Fig. 1A), containing all of the egg-laying activity, was further purified on a C18 Sep-Pak cartridge. The peptides were differentially eluted from the cartridge, using 20-30% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and 31-35% acetonitrile containing 0.1% TFA. The resulting samples were then fractionated by C18 reversed-phase HPLC (Fig. 1B,C). The major fraction, which eluted at 93 through 97 min (IIA in Fig. 1C), consistently induced egg laying when injected into sexually mature Aplysia (12 μg tested). Amino acid compositional and sequence analyses demonstrated that fraction II-A was a 36-residue peptide whose amino acid sequence was identical to the 36-residue sequence determined for the egg-laying hormone of A. californica (Table 1; 4), and to that predicted from nucleotide sequence analysis of an A. californica genomic ELH clone (23). The amount of ELH that consistently elicited egg laying (2.7 nmol) was comparable to that determined by Chiu and colleagues in A. californica (2.5 nmol; 4), and similar to the amount (2.5 nmol) predicted to be released from the A. californica bag cells during an afterdischarge (19). Approximately 3 μg of ELH was recovered from each pair of bag cell clusters. The calculated M_r, assuming COOH-terminal amidation (23), was 4384.

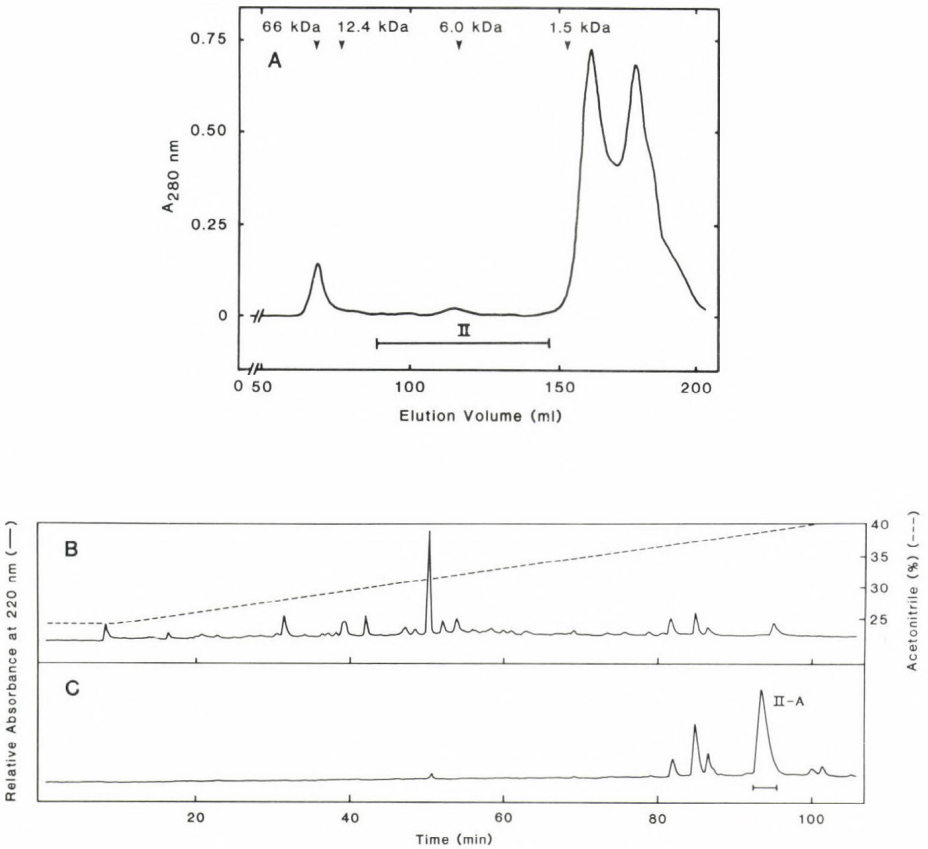


Fig. 1. Purification of A. brasiliana bag-cell peptides. A Bag-cell clusters were extracted in an acidic solution containing four peptidase inhibitors, and the extract chromatographed on a Sephadex G-50 column under acidic conditions. Molecular weight standards: bovine serum albumin (66 kDa); cytochrome c (12.4 kDa); aprotinin (6 kDa); bacitracin (1.5 kDa). B-C Pooled fraction II was applied to a C18 Sep-Pak cartridge and differentially eluted, using either 20-30% acetonitrile containing 0.1% TFA or 31-35% acetonitrile containing 0.1% TFA. Each of these samples was then applied to a C18 HPLC column (B and C, respectively) and a linear gradient of 0.1% TFA and acetonitrile containing 0.1% TFA was run. Only the peak labelled II-A in C had egg-laying activity.

Table 1. Comparison of the primary structures of atrial gland,

	1	2	3	4	5	6	7	8	9	10	11	12
A. brasiliana ELH[b]	Ile	Ser	Ile	Asn	Gln	Asp	Leu	Lys	Ala	Ile	Thr	Asp
A. californica ELH[b]	Ile	Ser	Ile	Asn	Gln	Asp	Leu	Lys	Ala	Ile	Thr	Asp
A. californica [Gln^{23},Ala^{27}]A-ELH[c]	Ile	Ser	Ile	Asn	Gln	Asp	Leu	Lys	Ala	Ile	Thr	Asp
A. californica [Ala^{27}]A-ELH[c]	Ile	Ser	Ile	Asn	Gln	Asp	Leu	Lys	Ala	Ile	Thr	Asp
A. californica A-ELH[d]	Ile	Ser	Ile	Asn	Gln	Asp	Leu	Lys	Ala	Ile	Thr	Asp
A. parvula ELH	Ile	Ser	Ile	Asn	Gln	Asp	Leu	Lys	Ala	Ile	Ala	Asp
Lymnaea stagnalis CDCH[e]	Leu	Ser	Ile	Thr	Asn	Asp	Leu	Arg	Ala	Ile	Ala	Asp

[a]Boxed residues indicate positions where the peptides differ from ELH.
[b]Determined by Chiu et al. (4).

PROTEOLYTIC PROCESSING OF ATRIAL GLAND PRECURSORS

Peptide studies (9,11,22) have provided evidence for the expression of three to four ELH-family genes in the exocrine atrial gland of Aplysia californica, including two A genes and one to two B genes. Information about the polyprotein precursors encoded by these genes and about the possible peptide products of the precursors have been provided by molecular genetic studies (10,23,24). Nevertheless, further peptide studies are required to show which of the predicted proteolytic cleavage sites are actually utilized in vivo, and whether or not more novel proteolytic sites might be utilized. Some of these studies are presented below. Knowledge of the peptide products of the atrial gland may provide a key to understanding its function in the animal.

Aplysia californica atrial gland peptides were extracted in an acidic solution containing two peptidase inhibitors (PMSF, Phe-Ala), and the extract was chromatographed on a Sephadex G-50 superfine column (Fig. 3A). The peptides in fractions D, E, and F were purified on a C18 Sep-Pak cartridge and were then subjected to C8 (Fig. 3B,C) or C18 (Fig. 3D) reversed-phase HPLC. Sequence analyses demonstrated that fraction D8 (Fig. 3B) contained three peptide complexes: A-AP/A-ELH, A-AP/[Ala^{27}]A-ELH, and A-AP/[Gln^{23},Ala^{27}]A-ELH (11). A mixture of these complexes (4.0 nmol) consistently induced egg laying when injected into receptive Aplysia. Further studies indicated that approximately 10-15% of these complexes were cleaved to yield smaller products, some of which were contained in fractions D3 and D7 (Fig. 3B). Compositional and sequence analyses of the

bag cell, and caudodorsal cell ELH-related peptides[a]

13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Met	Leu	Leu	Thr	Glu	Gln	Ile	Arg	Glu	Arg	Gln	Arg	Tyr	Leu	Ala	Asp	Leu	Arg	Gln	Arg	Leu	Leu	Glu	Lys
Met	Leu	Leu	Thr	Glu	Gln	Ile	Arg	Glu	Arg	Gln	Arg	Tyr	Leu	Ala	Asp	Leu	Arg	Gln	Arg	Leu	Leu	Glu	Lys
Met	Leu	Leu	Thr	Glu	Gln	Ile	Gln	Ala	Arg	Gln	Arg	Cys	Leu	Ala	Ala	Leu	Arg	Gln	Arg	Leu	Leu	Asp	Leu
Met	Leu	Leu	Thr	Glu	Gln	Ile	Gln	Ala	Arg	Arg	Arg	Cys	Leu	Ala	Ala	Leu	Arg	Gln	Arg	Leu	Leu	Asp	Leu
Met	Leu	Leu	Thr	Glu	Gln	Ile	Gln	Ala	Arg	Arg	Arg	Cys	Leu	Asp	Ala	Leu	Arg	Gln	Arg	Leu	Leu	Asp	Leu
Met	Leu	Ile	Val	Glu	Gln	Lys	Gln	Glu	Arg	Glu	Lys	Tyr	Leu	Ala	Asp	Leu	Arg	Gln	Arg	Leu	Leu	Asn	Lys
Ser	Tyr	Leu	Tyr	Asp	Gln	His	Trp	Leu	Arg	Glu	Arg	Gln	Glu	Glu	Asn	Leu	Arg	Arg	Arg	Phe	Leu	Glu	Leu

[c] Determined by Nagle et al. (11) and Rothman et al. (22).
[d] Predicted from nucleotide sequence analysis of an A. parvula bag cell genomic ELH clone (12).
[e] Determined by Ebberink et al. (7).

reduction and alkylation products of D3 (not shown) demonstrated that this fraction contained A-AP/A-ELH-(15-36), A-AP/[Ala^{27}]A-ELH-(15-36), and A-AP/[Gln^{23},Ala^{27}]A-ELH-(16-36) (summarized in Fig. 4A,B,C). The 14-residue NH_2-terminal fragment of A-ELH, which is identical to [Ala^{27}]A-ELH-(1-14) and [Gln^{23},Ala^{27}]A-ELH-(1-14), was independently isolated by Rothman et al. (21). Thus, in at least two of the complexes contained in D3, cleavage occurred at the Leu^{14}-Leu^{15} bond. The specificity of the atrial gland endopeptidase was thus similar to the vertebrate enzyme renin, which cleaves the Leu-Leu bond of angiotensinogen to generate Angiotensin I. It is not yet known whether the third complex contained in fraction D3 was also initially cleaved at the Leu^{14}-Leu^{15} bond, followed by aminopeptidase removal of Leu^{15} to generate A-AP/[Gln^{23},Ala^{27}]A-ELH-(16-36). A mixture of the three truncated complexes (35 nmol tested) did not induce egg laying when injected into sexually mature Aplysia, indicating that the NH_2 terminus of the 36-residue A-ELH-related peptides is necessary for biological activity. Compositional analysis of the reduction and alkylation products of fraction D7 (not shown) indicated that this fraction contained A-AP/A-ELH-(1-33) and A-AP/[Ala^{27}]A-ELH-(1-33) (summarized in Fig. 4A,B). The truncated form of the third complex, A-AP/[Gln^{23},Ala^{27}]A-ELH-(1-33), was not observed, but would not have been detected by compositional analysis if present in trace amounts. As in fraction D3, cleavage occurred at a Leu-Leu bond in these truncated complexes. Each of the intact complexes contained an additional Leu-Leu bond, at residues 11 and 12 of A-AP, but compositional analyses of other HPLC fractions indicated that cleavage did not occur at this site.

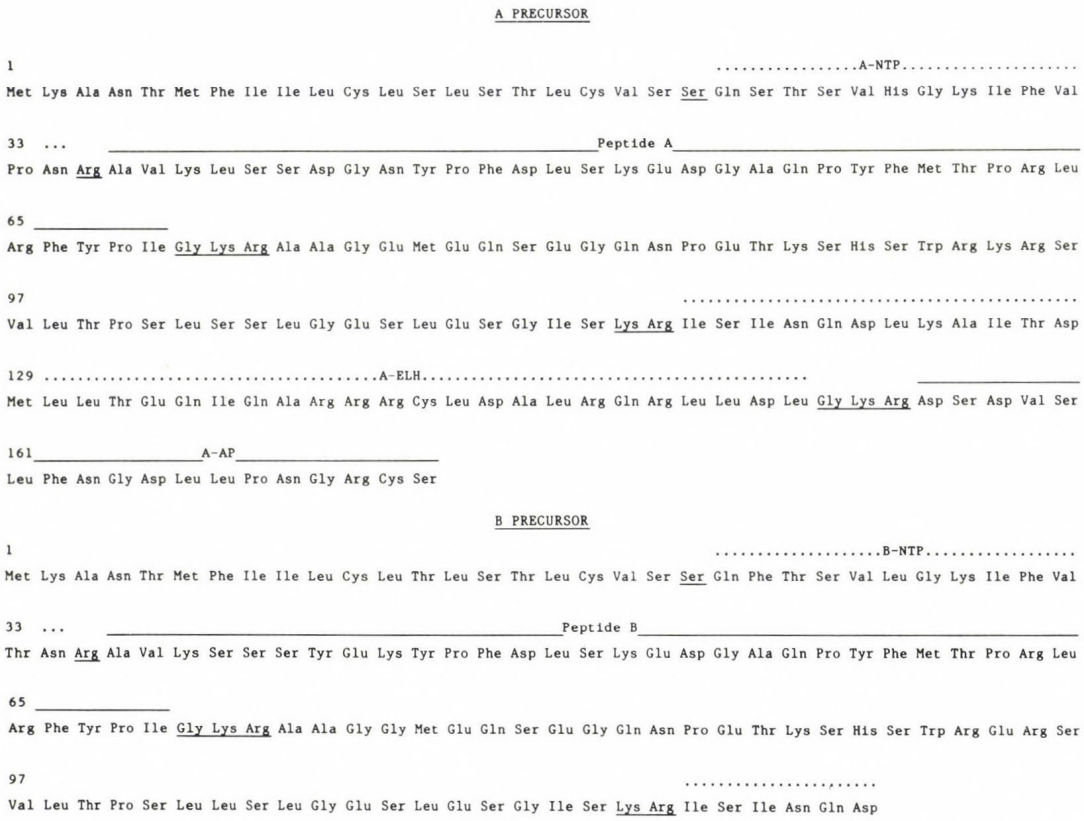
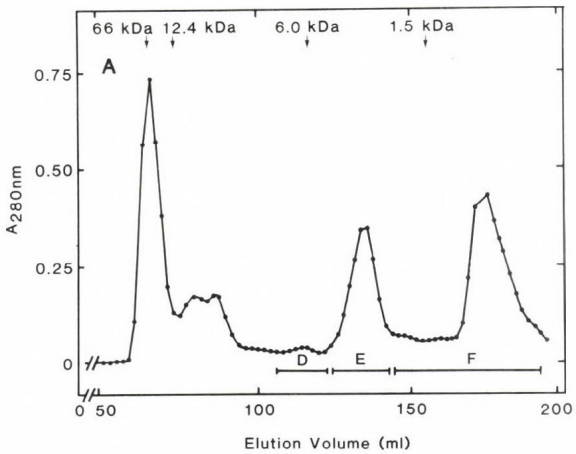


Fig. 2. Amino acid sequences of the A. californica atrial gland A and B precursors. The numbers above each sequence indicate residue position within each precursor. Major sites of proteolytic cleavage are underlined. Peptide sequences that have been isolated are labeled. The signal sequence comprises residues 1-21 in each precursor. The predicted precursor that encodes Ala^{27} -A-ELH is identical to the A precursor except at positions 72 and 143, where Lys replaces Arg and Ala replaces Asp.



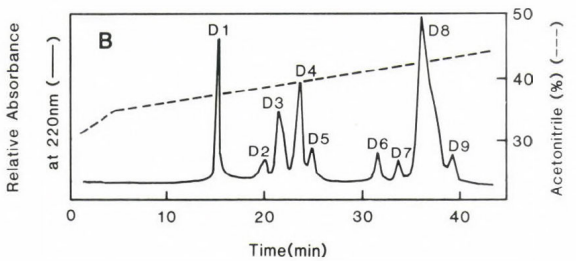
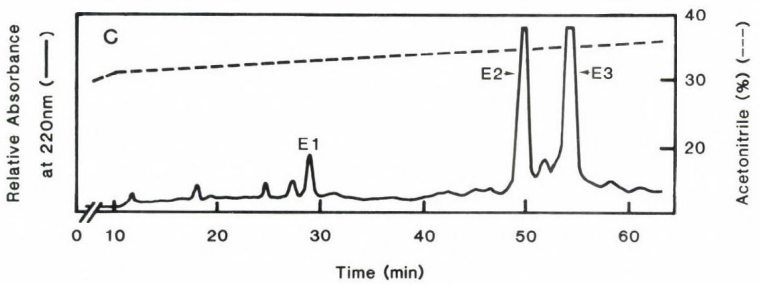
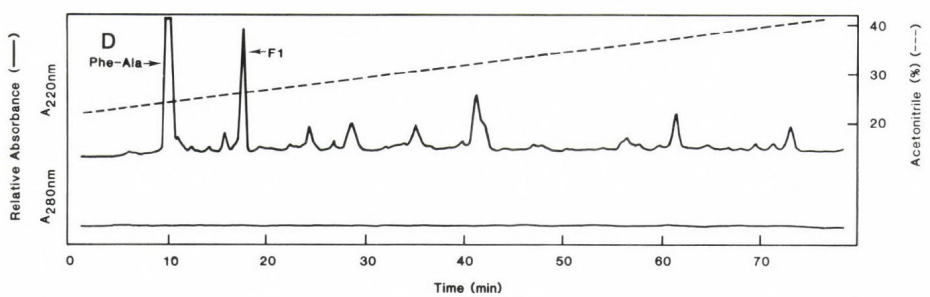


Fig. 3. Purification of A. californica atrial gland peptides. A Ten atrial glands were extracted in an acidic solution containing two peptidase inhibitors, and the extract fractionated on a Sephadex G-50 column. Molecular weight standards as in Fig. 1A. B-D Purification of fractions D (B), E (C), and F (D) by reversed-phase HPLC. The sample was applied to a C8 (B,C) or C18 (D) HPLC column and a gradient of 0.1% TFA and acetonitrile containing 0.1% TFA was then run.





Compositional analyses of HPLC fractions E2 and E3 (Fig. 3C) demonstrated that the compositions were identical to that of peptides B and A, respectively (9). Compositional analysis of fraction F1 (Fig. 3D) suggested the presence of two peptides: (a) a 13-residue NH_2-terminal peptide sequence (A-NTP) encoded by an atrial gland A gene cDNA clone (10) and a head ganglia A gene cDNA clone (24), and (b) a 13-residue NH_2-terminal peptide sequence (B-NTP) encoded by a genomic B clone (23). Attempts to separate the component peptides in F1 were unsuccessful, and sequence analyses of this fraction indicated that the NH_2 terminus was blocked. Fraction F1 contained one Glu/Gln residue, suggesting that the NH_2 terminus of A-NTP and B-NTP was a pyroglutamyl residue resulting from cyclization of the Gln following removal of the signal sequence of the nascent A and B precursors (Figs 2, 4).

The posttranslational processing events thought to occur in the atrial gland A and B precursors are summarized in Figure 4; the peptides that have been isolated to date (with the exception of [Gln^{23},Ala^{27}]A-ELH-(1-15) and A-AP/[Gln^{23},Ala^{27}]A-ELH-(1-33)) are labeled on the second and third lines for each precursor. The 173-residue A precursor (10) is processed to generate a 21-residue signal peptide, A-NTP, peptide A, a putative 42-residue sequence (which has not yet been detected in peptide studies), A-ELH, and A-AP. A second 173-residue A precursor (24) is processed to yield identical peptides, with the exception that [Ala^{27}]A-ELH, rather than A-ELH, is liberated. A-ELH and [Ala^{27}]A-ELH are each disulfide-bonded to A-AP. The complexes induce egg laying when injected into receptive Aplysia and are approximately equipotent with bag-cell ELH in this activity. A small proportion of each complex is further processed to yield A-AP/A-ELH-(15-36) or A-AP/[Ala^{27})A-ELH-(15-36) and A-ELH-(1-14). This processing is accompanied by a loss of egg-laying activity. However, since induction of egg laying is not likely to be the function of the exocrine atrial gland, it is not clear whether this processing represents an activation or inactivation step. A smaller proportion of each intact complex is processed to yield A-AP/A-ELH-(1-33) or A-AP/[Ala^{27}]-A-ELH-(1-33). Once again, the function of the processing step is not clear. The isolation of A-NTP provides the first direct evidence for the precise site of signal sequence cleavage in these precursors.

The 122-residue B precursor (23) is processed to generate a 21-residue signal peptide, B-NTP, peptide B, a putative 42-residue sequence, and a putative 6-residue ELH-related sequence. Neither the 42-residue nor the

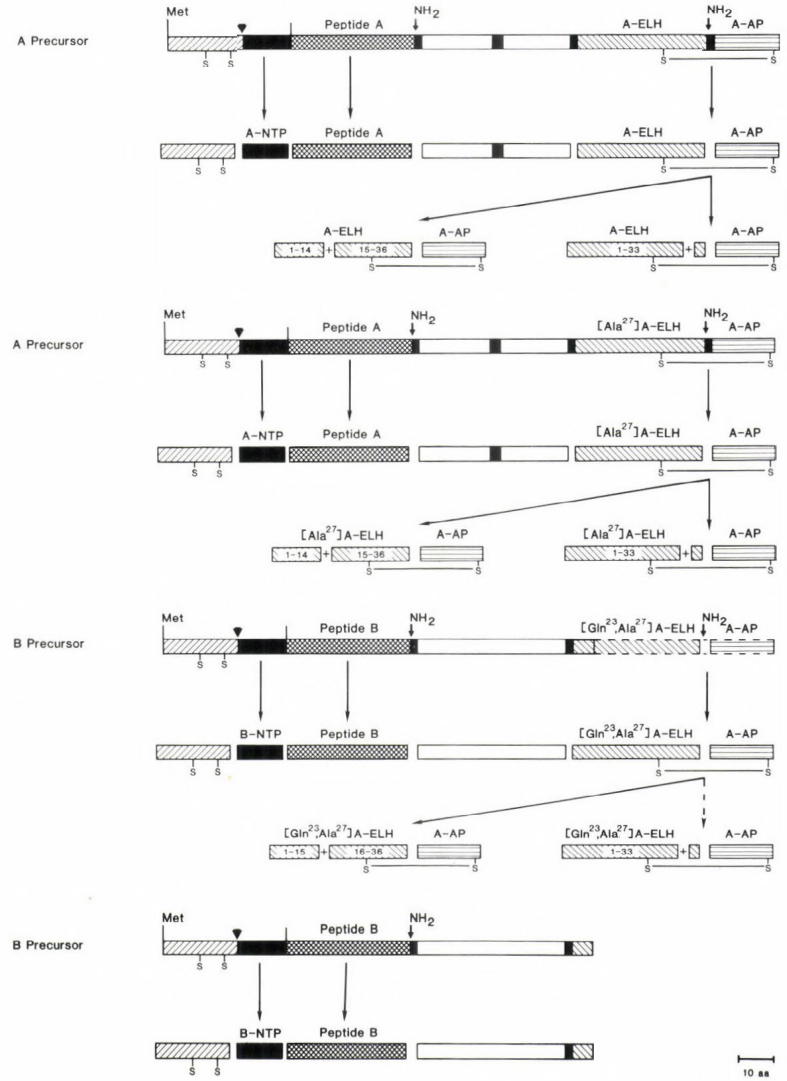


Fig. 4. Summary of the posttranslational processing of A. californica atrial gland A and B precursors. Homologous peptides have identical shading. An S indicates a Cys residue. Large arrows indicate the site of signal sequence cleavage. Vertical lines above the precursor indicate cleavage at single Arg residues. Black rectangles in the precursor show positions of potential mono-, di-, or tribasic cleavage sites. COOH-terminal amidation is indicated by an NH_2 written above an arrow. 10-15% of the intact complexes are cleaved to yield smaller products.

6-residue sequences have yet been detected in peptide studies. In contrast to A-ELH and [Ala^{27}]A-ELH, [Gln^{23},Ala^{27}]A-ELH is not encoded by any genomic or cDNA clone analyzed to date. The amino acid sequence of this peptide is identical to the sequence that could have been encoded by the characterized B gene (23), had a single base deletion (relative to the A gene) not occurred in the codon of residue 6 of the ELH-related sequence. Restriction enzyme mapping experiments have suggested that a second B gene exists (23). If this gene differs from the characterized B gene in the codon of residue 6 (that is, if the base deletion did not occur), this gene could encode the peptides in the A-AP/[Gln^{23},Ala^{27}]A-ELH complex (Fig. 4C). A small proportion of this complex is subsequently processed to A-AP/[Gln^{23},Ala^{27}]A-ELH-(16-36), accompanied by a loss of egg-laying activity, but the function of the processing step is still not clear. The isolation of B-NTP indicates where signal sequence cleavage occurs.

RELATED MOLLUSCAN EGG-LAYING HORMONES

The sequences of two other molluscan egg-laying hormones have been determined from either molecular genetic (Aplysia parvula, 12) or peptide (Lymnaea stagnalis, 7) studies, and each has significant sequence homologies with the A. brasiliana and A. californica ELH. Two genomic clones encoding the ELH of A. parvula (12) predict the synthesis of a precursor protein containing an ELH-related peptide, an AP-like peptide, alpha BCP, beta BCP, and a gamma-like BCP. The predicted 36-residue ELH-related peptide (Table 1) is 78% homologous to A. brasiliana and A. californica ELH, and, although not directly demonstrated, should induce egg laying when injected into all three species (18). The ELH of A. parvula is identical to A. brasiliana and A. californica ELH in 13 of 14 NH_2-terminal residues and in 11 of 12 COOH-terminal residues, but differs significantly from them between residues 15 and 24. These analyses suggest that egg-laying activity in Aplysia may be correlated with conservation of NH_2- and/or COOH-terminal regions of the molecule.

Although the ELH gene of A. californica appears to be expressed only in the bag cells and specific populations of central neurons (2,3,10,14,17,23,24), the ELH genes of A. parvula are also expressed in peripheral neurons along the length of the reproductive tract (12). Since A. parvula lacks a distinct atrial gland, and does not express genes encoding the A- or B-precursors, it is possible that these neurons function

to release ELH-related peptide products into the lumen of the reproductive tract. Whether or not the ELH-related peptides would be further processed in the peripheral neurons, as in the atrial gland, is not known.

The caudodorsal cell hormone (CDCH), which induces ovulation in the pulmonate gastropod Lymnaea stagnalis, is a 36-residue peptide (Table 1) which is 44% homologous to A. brasiliana and A. californica ELH (7). CDCH is identical to the Aplysia ELH-related peptides at 7 of 14 NH_2-terminal residues and at 5 of 12 COOH-terminal residues, perhaps suggesting that these conserved regions may be important for hormone binding to receptor sites. However, in contrast to the A. parvula ELH, Lymnaea CDCH is not likely to induce egg laying when injected into Aplysia (5).

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INDUCTION OF MATING BEHAVIOR IN APLYSIA BY FRESHLY DEPOSITED EGG CORDONS AND ATRIAL GLAND FACTORS

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Egg-laying activity in the marine mollusc Aplysia has been the focus of intensive investigation during the past 20 years and many aspects of this classic neuroendocrine system are now well understood. The bag cells, two clusters of neurosecretory cells located in the rostral portion of the abdominal ganglion, appear to be part of the final common pathway leading to egg deposition. These cells synthesize and release several biologically active peptides, including egg-laying hormone (ELH) and α-bag cell peptide(α-BCP) (4,11). ELH acts on the ovotestis to release eggs into the small hermaphroditic duct (12), while ELH, α-BCP and other bag-cell peptides affect neuronal activity in several central ganglia (6,14,15).

The bag-cell peptides are encoded by a single gene, known as the ELH gene. It belongs to a small family of genes, each of which encodes a polyprotein precursor containing sequences homologous to ELH and α-BCP. The ELH gene is expressed in the neuroendocrine bag cells but not in the exocrine atrial gland (4,11,13), while several other ELH-family genes are expressed in the atrial gland but not in the bag cells (5,7,10,13). In contrast to the bag-cell products of the ELH gene, the peptide products of the atrial gland genes have no physiologically defined function. To date, all that is known is that these products are secreted into the oviduct (1,3,8).

The experiments presented in this paper were designed to test whether or not atrial gland products might serve a pheromonal role in Aplysia. The results suggest that atrial gland factor(s), secreted onto the egg cordon as it travels through the oviduct, may induce copulatory behavior between individual Aplysia.

THE EFFECTS OF EGG LAYING ON REPRODUCTIVE BEHAVIOR

The first series of experiments was designed to investigate whether or not egg-laying activity by one Aplysia can affect the reproductive behavior(s) exhibited by a sexually mature conspecific with which it has been paired. Aplysia brasiliana (Rang) was used as the experimental animal since this species lays eggs and copulates more frequently than A. californica (9, and personal observations). Specimens of A. brasiliana were collected from the south Texas coast and were housed in individual perforated plastic cages in one of three large aquaria containing recirculating artificial seawater (ASW). All of the animals were sexually mature and could lay eggs spontaneously or in response to injections of atrial gland extract. Three criteria were used to select animals for each experiment: (a) the animal must not have laid eggs during the preceding 24 hr; (b) the animal must not have participated in a behavioral experiment during the preceding 24 hr; and (c) the animal must be paired with another Aplysia housed in the same aquarium. Once selected and paired, the animals were randomly assigned to one of the four treatments described below.

Sexually mature Aplysia californica (Cooper), purchased from Alacrity Marine Biological Services (Redondo Beach, CA), served as the source of atrial glands and large hermaphroditic ducts (LHD's) for the extracts used in these experiments. The atrial gland of A. californica was used because, compared to the atrial gland-like epithelium of A. brasiliana, it is a large, well-defined organ that is easily separated from other regions of the LHD (8). The peptides contained in the atrial gland and atrial gland-like epithelium are similar since: (a) extracts of the A. californica atrial gland induce egg laying when injected into either species; (b) extracts of the A. brasiliana atrial gland-like epithelium induce egg laying when injected into either species; and (c) polyclonal antibodies generated against peptides isolated from the A. californica atrial gland immunocytochemically label the atrial gland-like epithelium of A. brasiliana (8).

In each experiment, one Aplysia brasiliana was injected with a seawater extract of the atrial gland to induce egg laying (8), while three other Aplysia were treated in ways that would not induce egg deposition (i.e., injected with a seawater extract of the caudal LHD, posterior to the atrial gland; injected with an equal volume of ASW; or handled without

any injection). Each animal was then placed in a separate 4-liter beaker containing aerated ASW. An untreated animal was added to each beaker 60 min later, and reproductive behaviors were scored for each pair of animals at 10-min intervals for 270 min. Three categories of behavior were recognized: (a) copulation (no distinction was made between copulation as a male, a female, or a simultaneous hermaphrodite -- ie., as a male and a female); (b) egg laying by the non-treated animal; and (c) no reproductive activity.

All of the Aplysia brasiliana paired with an egg-laying animal copulated within the experimental time period, compared to an average of 75% of those paired with a non-laying animal (Table 1). The mean latency to the initiation of copulation was significantly shorter when Aplysia were paired with egg layers than with non-layers ($p<0.01$, one-way analysis of variance). Moreover, the differences in latency are underestimated in Table 1 since it was assumed, for purposes of calculation, that all animals failing to mate within the 270-min experimental period would have done so at 280 min. Since all of the non-copulating animals were found in the pairings with non-layers, the calculated mean latencies for these groups underestimate the true latencies to copulation.

None of the animals paired with either egg layers or non-layers laid eggs during the experimental period (Table 1).

THE EFFECTS OF TISSUE HOMOGENATES AND EXTRACTS ON REPRODUCTIVE BEHAVIOR

The second series of experiments was designed to investigate whether homogenates and extracts of specific regions of the reproductive tract could, when added to the bathing medium, mimic the effects of egg laying on reproductive behavior. As in the preceding series of experiments, Aplysia brasiliana were used as experimental animals, while A. californica served as a source of atrial glands and LHD's for homogenates and extracts. Five pairs of A. brasiliana, selected as outlined in the preceding section and randomly assigned to treatments, were used in each of the experiments. Two animals were placed together in a 4-liter beaker containing aerated ASW and then a test solution was immediately added to the beaker. Reproductive behaviors were scored for each pair of animals at 10-min intervals for 270 min. The five test solutions consisted of: (a) a seawater homogenate of the atrial gland; (b) an acidic extract of the atrial gland which had been lyophilized and resuspended in ASW; (c) a

Table 1. The effects of egg-laying activity by one Aplysia brasiliana on the reproductive behaviors exhibited by a sexually mature conspecific with which it has been paired (N = 20 for each treatment)

Treated Animal		Behavioral Response of Non-treated Conspecific			
		Copulation		Egg Laying	
Behavior	Treatment[a]	%[b]	Latency[c]	%[b]	Latency[c]
Egg laying	AG extract	100	69.5±10.6[d]	0	280[c]
Non-laying	LHD extract	80	149.0±21.3	0	280[c]
Non-laying	ASW	75	148.6±23.8	0	280[c]
Non-laying	No injection	70	162.5±21.5	0	280[c]

[a]Four treatments were used: (1) injection of atrial gland (AG) extract, the only procedure that induced egg laying; (2) injection of an extract of the caudal large hermaphroditic duct (LHD); (3) injection of artificial seawater (ASW); and (4) handling without any injection. The last three treatments did not cause egg laying and were used as controls.

[b]The percentage of non-treated animals exhibiting (or participating in) the behavior during the 270-min experimental period.

[c]Mean ± S.E.M. in min. When an animal did not exhibit the behavior during the experimental period, the latency was assumed to be 280 min for purposes of calculation.

[d]Relative to each of the non-laying (control) groups, egg-laying animals induced copulaton with a significantly shorter mean latency ($p<0.01$, one-way analysis of variance).

neutral (low ionic strength) extract of the atrial gland; (d) a seawater homogenate of the LHD; and (e) ASW. The results are summarized in Table 2.

A larger proportion of the animals exposed to homogenates or extracts of the atrial gland copulated during the experimental period than did those exposed to the two control solutions (homogenates of the LHD and ASW). The mean latencies to copulation were also significantly shorter for the atrial gland groups than for the control groups ($p<0.02$, one-way analysis of variance). As noted previously, the difference in mean latency to copulation between any of the atrial gland groups and the LHD

Table 2. The effects of the addition of tissue homogenates and extracts to the bathing medium on the reproductive behaviors exhibited by a pair of sexually mature Aplysia brasiliana (N = 25 for each treatment)

	Behavioral Response			
	Copulation		Egg Laying	
Treatment[a]	%[b]	Latency[c]	%[b]	Latency[c]
AG homogenate	84	107.6±12.4[d]	0	280[c]
AG acidic extract	92	111.2±13.8[d]	0	280[c]
AG neutral extract	84	122.4±16.8[d]	0	280[c]
LHD homogenate	52	201.2±17.7	0	280[c]
ASW	64	186.4±18.9	0	280[c]

[a]Abbreviations as in Table 1.

[b]The percentage of pairs of animals exhibiting (or participating in) the behavior during the 270-min experimental period.

[c]Mean ± S.E.M. in min. When an animal did not exhibit the behavior during the experimental period, the latency was assumed to be 280 min for the purposes of calculation.

[d]$p<0.02$ relative to the LHD homogenate and ASW (control) groups; one-way analysis of variance.

or ASW group is underestimated in Table 2 because of the larger numbers of non-mating pairs of Aplysia in the LHD and ASW groups, all of which were assigned a latency of 280 min for purposes of calculation. There were no significant differences in latency among the atrial gland groups ($p>0.05$; one-way analysis of variance).

None of the animals in any of the groups laid eggs during the experimental period (Table 2).

DISCUSSION

The experiments described in this paper demonstrate that Aplysia that are actively laying eggs are more likely to copulate with other Aplysia than are animals that are not laying eggs: there is a significantly shorter mean latency to the initiation of copulation when animals are paired with egg layers than when paired with non-layers. This decrease in mean latency to copulation can be mimicked by the addition of homogenates

or extracts of the atrial gland to the bathing medium; homogenates of the posterior LHD, which is composed predominantly of oviductal tissue (8), do not have similar activity. These results suggest that atrial gland factor(s) may be deposited on the egg cordon as it is transported through the oviduct past the atrial gland, and that these factor(s) may play a role in inducing or facilitating mating behavior. It is not yet known whether the factor(s) affect the egg-laying animal, making it more receptive to another Aplysia (90% of egg layers initially mated as females), or affect neighboring non-laying animals, making them more likely to initiate copulatory behavior. Experiments are in progress to try to distinguish between these possibilities.

Early experiments (2) showed that egg-laying Aplysia californica are attractive copulatory partners to other Aplysia, and that copulatory chains are more stable when one of the animals in the chain is laying eggs. These experiments suggested that there may be pheromonal inducers of copulation in Aplysia. Later experiments (16) showed that extracts of the rostral large hermaphroditic duct of Aplysia fasciata could, when added to the bathing medium, induce mating behavior in this species. Our experiments confirm and extend these studies by localizing the active factor(s) to the atrial gland, an exocrine organ of unknown function secreting into the oviduct. Our studies have also shown that the active factor(s) are stable when extracted in either acidic or neutral media, information that is important for ongoing experiments to isolate and chemically characterize the factor(s) (e.g., 7).

It remains to be established whether or not the factor(s) influencing mating behavior are products of the ELH-family genes expressed in the atrial gland. If they are, these observations raise the interesting possibility that subsets of gene families, expressed in a tissue-specific manner in Aplysia, may control different subsets of reproductive activity. Thus, peptide products of the ELH gene, expressed in the neuroendocrine bag cells, would coordinate egg-laying behaviors within an animal, while peptide products of other ELH-family genes, expressed in the atrial gland, would coordinate mating behavior among animals.

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DISCUSSION

BRUSSAARD, A.B.: You said during your lecture that whenever a snail would lay eggs, other animals got interested, and responded by exerting reproductive activity, either copulating or laying eggs themselves near the first snail. You also stated that atrial gland-peptides upon injection effectively induce egg-laying in Aplysia. I wonder whether in any of your experiments - in which you replaced egg-laying snails by fresh pairs of animals - the latter animals ever responded by laying eggs instead of copulating?

BLANKENSHIP, J.E.: The experiments I discussed today were of two types: 1. in some experiments we examined whether a "naive" animal added to an egg-laying animal would copulate sooner or more often than an equivalent animal added to a non-egg-laying animal; and

2. we also did experiments in which two "naive" animals were added to a beaker containing a freshly laid egg mass. An animal actively laying eggs, and a fresh egg mass were essentially equally capable of inducing copulation. We also have preliminary evidence that an animal which has just laid eggs is not an effective stimulus for inducing copulation.

LUKOWIAK, K.: The data concerning latency to copulate in the 4 groups of animals appear to be quite different between your two sets of experiments. In the first group the latency to copulate in the atrial gland injection group was around 50 ms; whilst in the 2nd atrial gland injection group the latency was 100 ms. Please explain.

BLANKENSHIP, J.E.: There is considerable variability in this (and other) factor. We were careful only to compare latencies within the same study.

LUKOWIAK, K.: Janet Leonard has demonstrated rather convincingly that Aplysia and Navanax alternate their sexual roles. Do the "pheromones" from the atrial gland cause an animal to choose one sexual role over the other?

BLANKENSHIP, J.E.: We have no convincing evidence that Aplysia alternate sexual roles in any systematic way. Switzer-Dunlap in Hawaii has similar data on other species of Aplysia. The only time sex role is predictable if copulation occurs with an egg-laying animal. Such an animal is constrained by functional anatomical considerations to copulate only as a female.

ROUBOS, E.: In L. stagnalis, cells expressing CDCH-like genes have been found not only in the brain but also in accessory sex glands including the oothecal gland, which contributes to the formation of the egg capsule. Have you tested a possible stimulatory effect of the secretory products of accessory sex glands of Aplysia on copulation?

BLANKENSHIP, J.E.: The only parts of the reproductive tract we have tested for egg laying activity are the atrial gland and two parts of the large hermaphroditic duct (LHD): that part of the LHD rostral to the atrial gland, and that part of the LHD caudal to the atrial gland up to the so-called accessory genital mass (=albumin gland). Only the atrial gland has egg-laying activity.

SAKHAROV, D.A.: Is the atrial gland an exclusively exocrine organ or, maybe, its active factor(s) act as hormone(s) as well?

BLANKENSHIP, J.E.: Yes, work from our lab and from that of Steve Arch have clearly shown this to be the case.

Symposia Biologica Hungarica 36 (1988)

THE EFFECT OF NEUROPEPTIDES ON ISOLATED NERVE CELLS (STRETCH RECEPTOR NEURONS OF CRAYFISH): INTERACTION WITH OTHER TRANSMITTERS

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INTRODUCTION

It is by now well known that a number of neuropeptides effectively alter the excitability of nerve cells to which they are applied. The mammalian neurohypophyseal peptides vasopressin, oxytocin and related cyclic nonapeptides occur in neurons of many brain regions (see review by Sofroniew, 1985) and there is now impressive evidence that these peptides serve as neurotransmitters in mammals (e.g. Buijs, 1983; Riphagen and Pittman, 1986) and in molluscs (e.g. Goldberg et al., 1987).

The discovery of vasopressin-like peptides in the nervous system of arthropods (see Remy, 1982) prompted us to analyze the action of cyclic nonapeptides and some synthetic derivatives on arthropod neurons. The large neurons of crayfish stretch receptor organs have already played a major part in the discovery of the transmitter role of y-aminobutyric acid (GABA) (Florey, 1953; Bazemore, Elliott and Florey, 1957; Craelius and Fricke, 1981). These neurons can be isolated together with the muscle fiber to which they are attached, and by holding the muscle fiber with fine micromanipulated forceps, the receptor neuron can be placed in the stream of a superfusion system which permits rapid and controlled application of various test substances (Iwasaki and Florey, 1969). Such preparations are viable for many hours and permit both extracellular and intracellular electrical recording.

Crayfish stretch receptor neurons respond to GABA and to dopamine, mainly with inhibition (Bazemore et al., 1957; Edwards & Kuffler, 1959; Iwasaki and Florey, 1969; McGeer, McGeer and McLennan, 1961). These neurons thus offer an opportunity of studying the interaction of neuropeptides with the effects of other transmitter substances.

MATERIALS AND METHODS

Initial investigations mainly involving extracellular recording used crayfish of the species Astacus leptodactylus, most experiments, however, were conducted with Orconectes limosus. The animals were obtained commercially and were maintained in large tanks supplied with running lake water at a temperature of $12^{o}C$.

The methods of preparing the stretch receptor organs have been described in detail elsewhere (Florey and Elliott, 1959). A technique of superfusing the isolated receptor neurons and of intracellular recording has also been fully described (Iwasaki and Florey, 1969). We have followed this method with some modifications: instead of a bridge circuit we made use of the single electrode current clamp technique (Axoclamp II) in order to inject current into the cell for measurements of input resistance and for changing the membrane potential. In our investigations we have used the slowly adapting receptor neuron.

The saline medium used had the following composition: NaCl 200, KCl 5.4, $CaCl_2$ 13.5, and $MgCl_2$ 2.6 mM. In some experiments the pH was maintained at 7.2 with 10 mM HEPES buffer, but in general it was found unneccessary to buffer the saline. Drugs and peptides were diluted in the particular saline used prior to the experiment. Solutions were applied by gravity flow. The various reservoirs were connected to a miniature switching manifold which in turn was connected to a cooling coil within the water jacket of the preparation bath (temperature $12^{o}C$). The preparation chamber itself was very shallow with a fluid depth of not more than 3 mm. The opening of the cooling coil (inner diameter 1 mm) protruded horizontally about 2 mm into the chamber, just 1 mm above the transparent chamber floor. The chamber volume was about 1 ml. The nerve cell was positioned close to the orifice and was thus subjected to rapid superfusion. About 2.5 ml passed over the neuron in one minute.

The axon of the receptor neuron was held in a suction electrode. The muscle fiber was slightly stretched to result in a neuronal discharge rate of about 2 impulses per second. The extracellularly recorded axon spikes were amplified (a.c.) and the signals were fed into a rate meter. For extracellular recording the analog d.c. signal of the rate meter was superimposed on the a.c. amplified spikes so that the baseline of the recording is a measure of rate of firing.

Muscle length was monitored with the aid of a potentiometer coupled to the micrometer used to stretch (or relax) the muscle fiber. A four-channel chart recorder (Brush-Gould 2400) was used in conjunction with a storage oscilloscope.

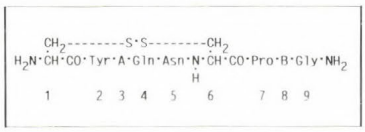
For intracellular recording we generally used 3 M KCl-filled microelectrodes with tip diameters giving a resistance between 10 and 50 MOhm.

GABA, dopamine-HCl, naloxone-HCl, metoclopramide-HCl, and ergonovin maleate were purchased from Sigma, sulpiride from Delagrange, Arpajon.

The cyclic nonapeptides used were arginine-vasopressin (AV), lysine-vasopressin (LV), and oxytocin (OT); all were purchased from Sigma. The structure of these compounds is shown in Fig.1.

We are grateful to Prof. W.H. Sawyer, Columbia University, for gifts of several derivatives of these cyclic nonapeptides. Their structure is shown in Fig.2.

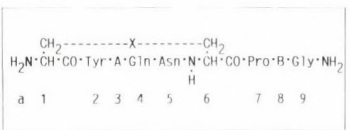
Cyclic Nonapeptides



	3		8	
AV (arg-Vasopressin)	Phe		Arg	
LV (lys-vasopressin)	Phe		Lys	
OT (oxytocin)	Ile		Leu	

Figure 1

Derivatives of Cyclic Nonapeptides



	X	a	2	3	4	8
HOTOT 1-(L-2-OH-3-MPA*)4-Thr-OT	S·S	OH	Tyr	MPA	Thr	Leu
HOOT 1-(L-2-OH-3-MPA*)-OT	S·S	OH	Tyr	MPA	Gln	Leu
d-VDAVP 1-deamino-(4-Val, 8,-D-Arg)-VP	S·S	$C(CH_3)_2$	Tyr	Phe	Val	D-Arg
dPTOT (1-deaminopenicillamine, 4-Thr)-OT	$S·CH_2$	$C(CH_3)_2$	Tyr	Ile	Thr	Leu
dPOMeOT (1-deaminopenicillamine, 2-O-Me-Tyr)-OT	$S·CH_2$	$C(CH_3)_2$	Tyr O-Me	Ile	Gln	Leu
dPOMeVT (1-deaminopenicillamine, 2-O-Me-Tyr)-VT	$S·CH_2$	$C(CH_3)_2$	Tyr O-Me	Phe	Gln	Arg

* mercaptopropanoic acid

Figure 2

RESULTS

Effects of GABA and of DA

Applied in low concentrations of 10^{-6} M, GABA causes a depolarization and a slight increase of membrane resistance (Florey and Rathmayer 1986). With higher concentrations of GABA (10^{-5} M or higher) the result is either a depolarization or a hyperpolarization; both are accompanied by a large decrease of membrane resistance (see Iwasaki and Florey, 1987). Both GABA effects were blocked by picrotoxin (PTX).

When superfusion with the higher GABA-concentration is begun, the neuron usually responds with transient excitation characterized by a slight depolarization and an increase in firing rate. This is followed by an inhibition, characterized by a hyperpolarization and cessation of spike discharges. On washing with normal saline, the neuron again shows transient depolarization and a supernormal rate of firing. Fig. 3 shows an example.

DA in concentrations above 10^{-7} M generally causes a slight hyperpolarization accompanied by a slight decrease of membrane resistance. This hyperpolarization is found even with neurons which respond to application of higher concentrations of GABA with depolarization. The application of DA results in reduction and cessation of spike discharges. When the superfusion with DA is continued for several minutes, the neuron usually starts to fire again and impulse rate increases well above the resting rate. This state of excitation is characterized by an increase of membrane resistance above resting values. The membrane resistance remains elevated even after washing and it may take up to an hour before the original conditions are restored. Both the inhibition and the excitation caused by DA are unaffected by PTX but are readily blocked by metoclopramide, ergonovin, and sulpiride. Examples are shown in Fig. 4.

During inhibition by GABA or DA the neurons remain sensitive to stretching. As is shown in Fig. 5, brief stepwise extensions of the muscle fiber result in spike discharges of the inhibited neuron.

Effects of nonapeptides

With the exception of lysine vasopressin, which was found to be without effect, all nonapeptides tested cause an inhibition of impulse discharge. The relative potency of the different compounds tried is shown in Table 1. On a molar basis,

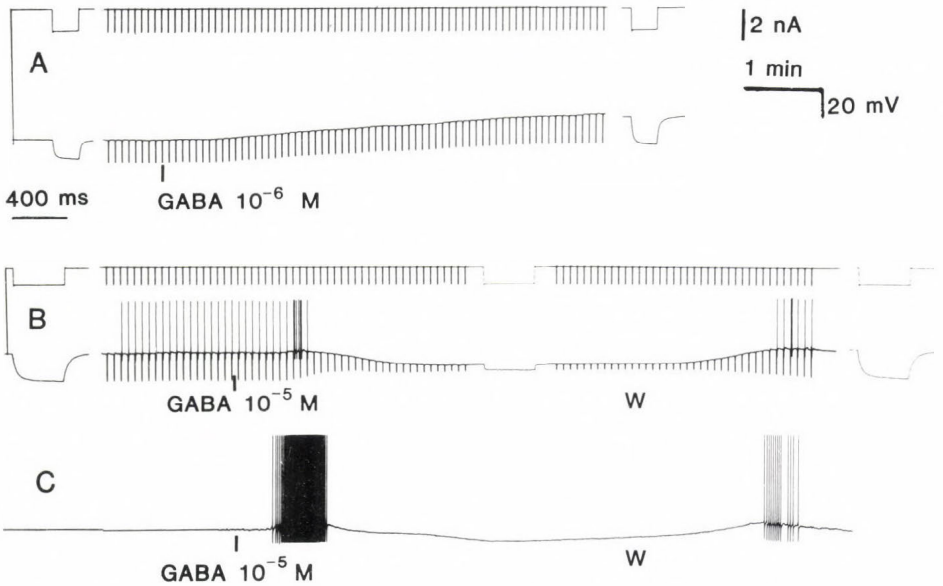


Figure 3. A: low concentration of GABA causes depolarization and an increase of membrane resistance. Upper channel = current pulses, lower channel = potential change. B: GABA in higher concentration causes hyperpolarization and marked decrease of membrane resistance. Same cell as in A; note transient excitation at beginning and end of GABA-application. Presentation as in A. C: in a 'silent' neuron GABA application causes transient depolarization and excitation at beginning and end of superfusion but during GABA application there is hyperpolarization.

Table 1. Relative potency* of cyclic nonapeptides

LVP	0
d-VDAVP	1
OT	100
AVP	1000
d-PTOT	2000
HOTOT	4000
HOOT	4000
d-POMeOT	6000
d-POMeVP	8000
Subst.P	0

*Causing complete inhibition of impulse discharge of crayfish stretch receptor neuron. Reciprocal of normalized threshold concentration.

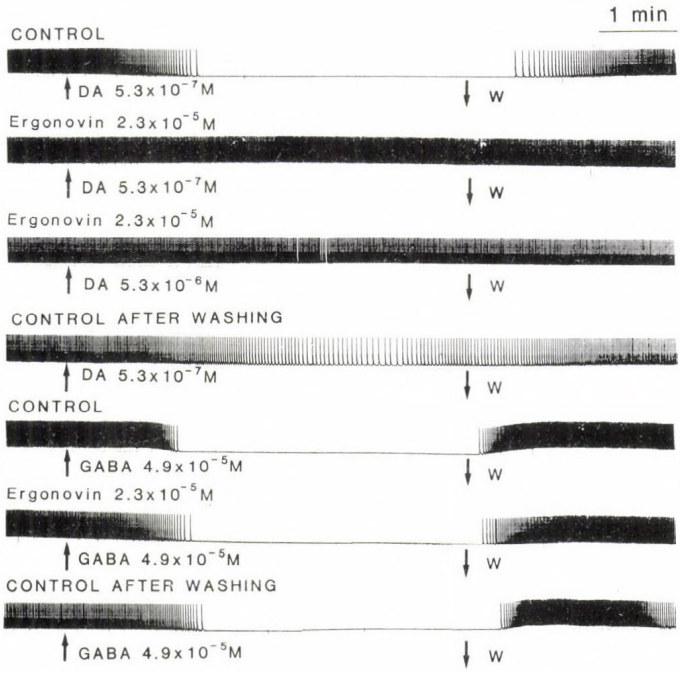


Figure 4. Extracellular recording. Both DA and GABA cause inhibition. The effect of DA is blocked by ergonovin, that of GABA is not.

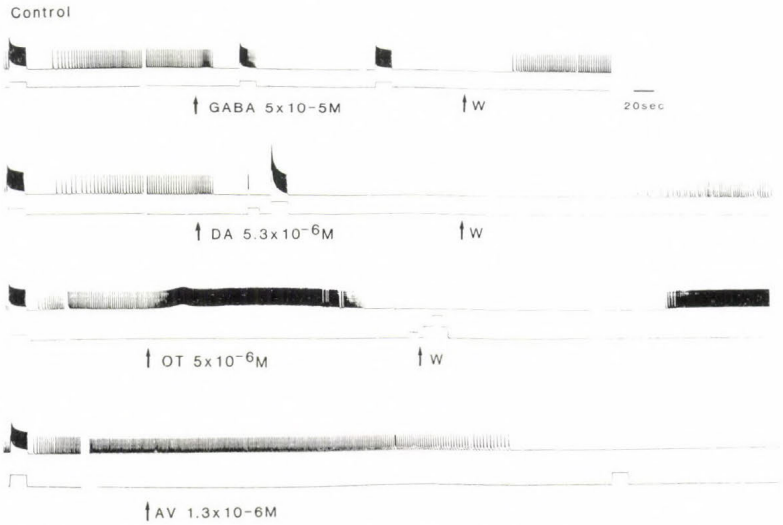


Figure 5. Extracellular recordings of spike discharges (upper channels) and of muscle length (lower channels). During inhibition by GABA and by DA stretching causes excitation; during inhibition by OT and AV mechanical sensitivity is lost. Note transient excitation with OT (not present with lower concentrations).

all of the effective peptides were 2 to 5 orders of magnitude more effective than GABA in causing inhibition. In several preparations we found this inhibition to be preceded by a transient, often very strong excitation. During inhibition, the preparations become completely insensitive to stretching. Examples are shown in Fig. 5. With intracellular microelectrodes it was seen that the inhibition is accompanied by a decrease of membrane resistance in most cases, but in some cells there is a slight increase; the membrane potential shows no significant change. Spike height progressively declines (see Fig. 6).

The receptor neurons often respond to hyperpolarizing current pulses with off-excitation: as the current pulse terminates the membrane depolarizes beyond the resting potential and this gives rise to one or more spikes. Under the influence of the nonapeptides, these off-effects become progressively reduced, until only abortive "off-spikes" can be recorded which no longer give rise to conducted nerve impulses. A few minutes later all active responses cease. The neurons remain depressed for some time after washing, the duration of this depression depends on the concentration of the peptide. During recovery, the ability to spike is restored when the cells are slightly hyperpolarized by current injection. Examples are shown in Fig. 7.

Many preparations were found which responded to peptide application with an initial strong depolarization. As is illustrated in Fig. 8, this depolarization is accompanied by a reduction of membrane resistance. The depolarization, which may reach 35 mV, inactivates the spike generation. Later, the membrane resistance increases above the resting value and this increased membrane resistance persists long after washing. The membrane potential slowly returns to normal and the neuron then fires. Other preparations, however, did not show this excitatory response (see Fig. 5).

Repeated application of all of the effective nonapeptides resulted in desensitization. None of the peptides tried can be regarded as a blocking compound.

The inhibitory effect of the nonapeptides was not affected by PTX (applied in concentrations as high as 10^{-4} M), but were prevented by naloxon (10^{-5} M). This drug did not prevent the excitatory effects.

Preparations that had been exposed to repeated applications of nonapeptides progressively lost their responsiveness to stretching even though they recovered the ability to fire repetitively.

Interaction with GABA and DA effects

Preparations which had recovered from a single effective dose of one of the nonapeptides showed greatly increased sensitivity to the inhibitory action of

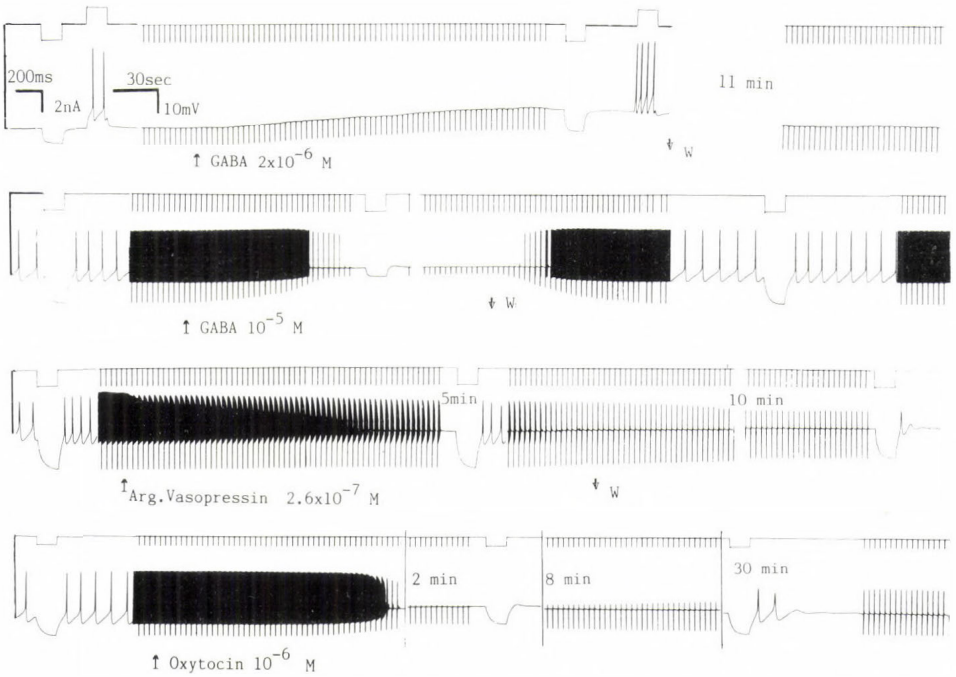


Figure 6. Effects of low and high concentration of GABA, and of AV and OT on membrane potential and resistance of same neuron. Upper channel of each recording = current pulses, lower channel = membrane potential. Note gradual decline of spike height under the influence of the peptides.

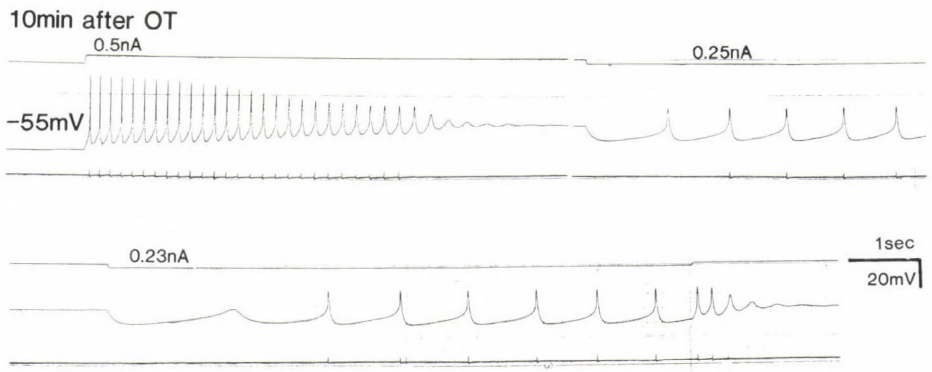


Figure 7. After OT 10^{-7} M, depolarization causes time dependent depression of spike discharges; this can be relieved by repolarization. Upper channels = injected current, middle channels = membrane potential, lower channels = extracellularly recorded axon spikes.

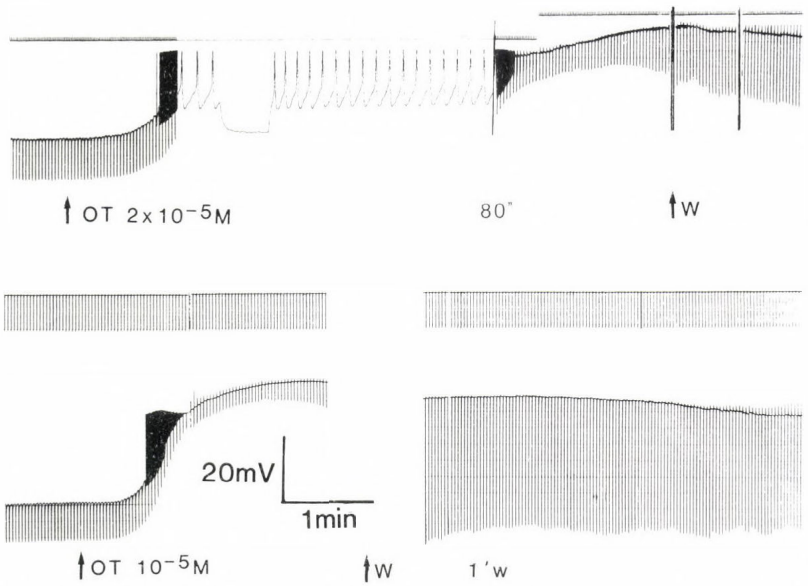


Fig. 8. Strong depolarization caused by OT seen in two preparations. Top trace = 1 s time marks, second trace = intracellular recording showing effect of injected current pulses (not shown). Third trace = current pulses of 2 nA; baseline indicates membrane potential. Bottom trace: recording of membrane potential.

GABA. No excitatory responses to GABA were seen. The sensitivity to DA, on the other hand, was greatly diminished or was lost altogether.

DISCUSSION

The sensitivity of the crustacean stretch receptor neurons to nonapeptides (and to their derivatives) suggests that these neurons are equipped with appropriate peptide receptors. Indeed, the ineffectiveness of lysine-vasopressin, and the difference in the activity of the other nonapeptides which covers a range of nearly two orders of magnitude (see Table 1), suggests considerable specificity of the receptor molecules. The blocking action of higher concentrations of naloxon, on the other hand, may represent an unspecific action of this drug - unless the receptors are also sensitive to opioids, a possibility which we have not yet explored.

In contrast to findings reported from experiments on vertebrates (Sawyer et al., 1981; Manning and Sawyer, 1983, 1984; Mühlethaler et al., 1983; Charpak et

al., 1984), none of the nonapeptide derivatives (see Fig. 2) can be classified as a receptor blocker. All of them were found to be agonists. In this regard the crustacean nonapeptide receptor(s) appear(s) to differ from the vertebrate receptor types.

The earlier reports of the occurrence of nonapeptides in the crustacean nervous system (see Remy, 1982) in conjunction with the sensitivity of crustacean neurons to such peptides suggests that nonapeptides play an important regulatory role in these animals. The drastic effects we report here may not be typical of the natural function of nonapeptides which may be far more subtile. Undoubtedly, these compounds affect neuronal excitability. More interesting, however, is the observation that such peptides can alter the responsiveness of neurons to other transmitters. It will be the task of future research to elucidate the mechanisms by which these effects are achieved. Among the possibilities are (i) a change of receptor affinity to a particular transmitter, (ii) a change in the coupling of receptors to ion channels, (iii) a change of the turnover of receptors, and (iv) a change in the turnover of ion channels. The observation that after peptide application the membrane resistance greatly increases suggests loss of certain ionic channels. It is quite possible that these are the same channels that are normally activated by DA or inactivated by low concentrations of GABA (which cause a resistance increase).

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NEUROMEDIATORS IN THE CNS OF THE FRESHWATER SNAIL PLANORBIS CORNEUS: LOCALIZATION AND EFFECTS ON THE ADENYLATE CYCLASE SYSTEM OF SPECIFIC GANGLIA

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INTRODUCTION

The nervous system of the freshwater gastropod Planorbis corneus (Planorbarius corneus) has been utilized by us as a model for morpho-functional studies. In particular, an analysis was carried out of the nervous network underlying the tentacle movements which were shown in a behavioural study to be modifiable by simple forms of learning (Biral et al. 1978). Some involved sensory cells and motoneurons have been characterized in the central and peripheral nervous systems (Sonetti et al. 1982a, b). More recently, we started a neurohistochemical survey of the central ganglia in order to identify the neuromediators (Sonetti 1984, Sonetti et al. 1986) and biochemical studies with the aim of clarifying the mechanisms by which such neuroactive substances might exert their physiological action (Sonetti et al. 1987). Since it is well known that many neurotransmitters might act indirectly through a second messenger system and because it has been demonstrated that cyclic AMP might play a fundamental role in plastic changes related to learning processes (Kandel and Schwartz 1982), we have investigated the effects of neuroactive substances on the cyclic AMP system. In this paper we report about the histo- and immunohistochemical localization in the CNS of P. corneus of two monoamines, serotonin (5-HT) and dopamine (DA), and two biologically active peptides FMRFamide (FM) and Substance P (SP). While it is well known that monoamines exert their effect on the invertebrate neurons by regulating the intracellular cyclic

AMP levels (Brunelli et al. 1976, Belardetti et al. 1982), few and contradictory reports are available on the molecular events underlying the physiological effects of neuropeptides. This large family of neuroactive substances has been intensively studied only in the last few years. The characteristics of these substances, i.e. the direct synthesis by mRNA, the wide variety of their roles, and the multiple functions they often assume, make them formidable candidates for an involvement in transient or profound long-lasting physiological changes. Their postsynaptic effects might be mediated by changes in membrane ion flux, perhaps by an intracellular enzymatic cascade, sometimes referred to as a second messenger (Brezina et al. 1987a, b).

In order to verify if cyclic AMP could mediate the action of such neuroactive substances in P. corneus, we have studied their effects on the adenylate cyclase system of selected ganglia of this snail.

MATERIALS AND METHODS

For this investigation adult specimens of P. corneus (shell size 20-30 mm) collected in a canal near Modena (Italy) have been utilized.

Histo- and immunohistochemistry

The whole ganglionic ring including buccal ganglia was quickly dissected under stereomicroscope. For the visualization of monoamines we used the slightly modified SPG method (de la Torre and Surgeon 1976). For immunohistochemical peptide detection the ganglia were fixed overnight at room temperature in GPA. 10 µM paraffin serial sections have been utilized. After an overnight incubation in a moist chamber with the primary antiserum the slides were immunocytochemically stained with the biotin-avidin system (BAS) (Vector Lab., Burlingame, CA) following the original Hsu schedule (Hsu et al. 1981) or by indirect fluorescence (FITC-conjugated secondary serum). Prior to

staining, the endogenous peroxidase activity was blocked by incubating the slides in 7.5% H_2O_2 and then in 2.28% periodic acid, 5 min each. For this research the following antisera were employed (dilutions in parentheses): a-SP monoclonal antibody (1:400) raised in rat (C. Milstein and C. Galfre, Lab. Mol. Biol. H.R.C., Hills Road, Cambridge, UK); a-FM antibody (1:500/1000) raised in rabbit (C.R.B., Cambridge, UK). All the antisera were dissolved in 0.3% Triton X-100 in PBS, pH 7.4. The specificity of the reactions was checked by omitting the primary antibodies or preadsorbing them in the liquid phase with the homologous antigen except the monoclonal a-SP tested on beads of activated Sepharose 4B-CNBr, coupled to synthetic SP (USB-UK). Each antiserum was tested on 4-10 ganglionic rings.

Adenylate cyclase assay

Adenylate cyclase activity was assayed in homogenates prepared from Cerebral Ganglia Complex (CGC), Pedal Ganglia Complex (PGC) and Viscero-Parietal Complex (VPC), according to the method of Clement-Cormier et al. (1975), as previously described by Sonetti et al. (1987). Proteins were determined according to Lowry et al. (1951).

RESULTS

The CNS of P. corneus consists of 9 ganglia arranged in a perioesophageal ring, as well as a pair of buccal ganglia situated anteriorly on the buccal mass and joined to the cerebral ganglia by two thin connectives. Orange-red and white (neurosecretory) pigmented neurons are visible in vivo beneath the connective tissue sheath.

5-HT and DA distribution

In Fig. 1 a distribution diagram for specific fluorescence related to 5-HT and DA is depicted.

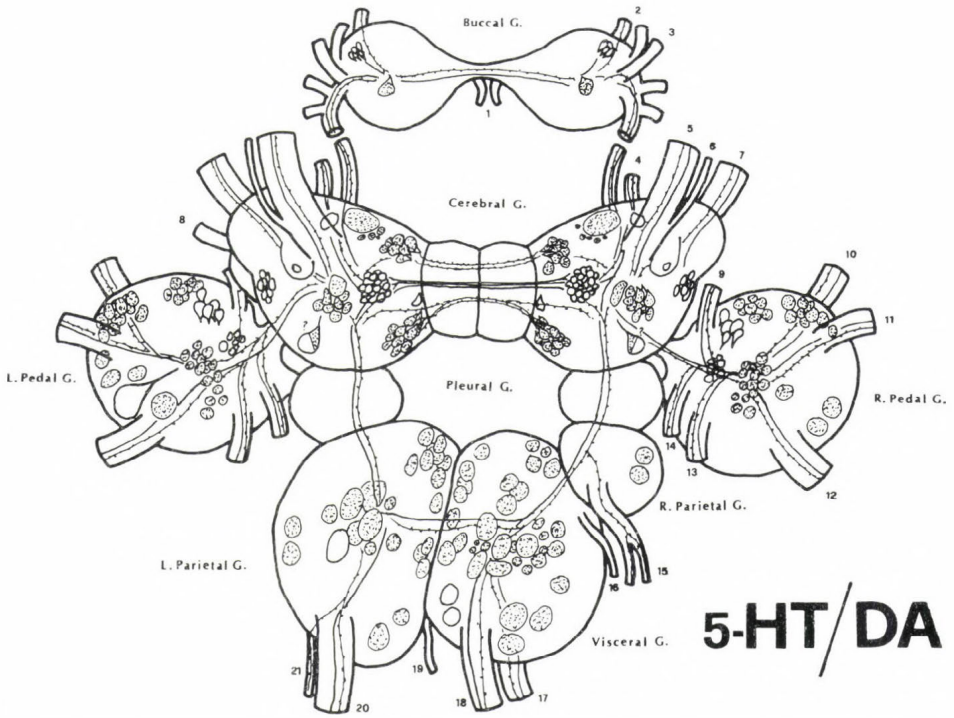

Fig. 1. Diagram of the dorsal surface of the circumoesophageal ganglia of P. corneus showing the position of cells and fibres exhibiting a specific fluorescence after glyoxylic acid treatment. Dotted cells and rough fibres contain 5-HT, open cells and smooth fibres contain DA. 1-21 nerves: 1 radular, 2 gastric, 3 pharyngeal, 4 superior frontal lip, 5 tentacular, 6 optic, 7 median lip, 8 penial, 9 superior cervical, 10 superior pedal, 11 median pedal, 12 inferior pedal, 13 columellar, 14 inferior cervical, 15 right pallial, 16 cutaneous pallial, 17 intestinal, 18 anal, 19 genital, 20 left internal pallial, 21 left external pallial.

By using the SPG method a majority of yellow fluorescent (5-HT-containing) and in smaller number blue-green fluorescent (DA-containing) cells are revealed in all the ganglia except the pleural ones. A diffuse network of fluorescent intermingled fibres is observed in all the central neuropils where the processes often assume a beaded appearance. From here bundles of fluorescent fibres cross the commissures and connectives and reach the major nerve trunks. In spite of the abundance of DA-containing fibres, the blue-green fluorescent neurons are rela-

tively few. They are found in symmetrical groups in the buccal, pedal and cerebral ganglia. The most numerous DA cluster (15-20 cells, 10-15 µm) and a pair of giant neurons (120-140 µm) that show both fluorescences are observed on the ventral side of these last ganglia (Fig. 2B). In the left pedal ganglion, besides the Giant Dopaminergic Neuron (GDN), a symmetrical group of cells on the ventral surface is revealed (Fig. 2C). Only few scattered cells are found in the visceral and right parietal ganglia. Besides the pair of Giant Serotonergic Neurons, at least three yellow fluorescent cell clusters lay in symmetrical position in the cerebral ganglia. Two of these are dorsally located and send the processes into the commissure (Fig. 2A). In the buccal ganglia a pair of yellow neurons are present but the greater number of serotonergic cells is found in the pedal and visceroparietal ganglia. The yellow cells are medium to large in size (30-200 µm) and also the fluorescence intensity of these cells varies considerably. In the pedal ganglia 5-HT cells predominate on the ventral side close to the commissure while in the visceral and left parietal ganglia the yellow cells are mainly located on the dorsal surface and their adjacent borders. Some weak yellow fluorescent cells are found also in the right parietal ganglion (Fig. 1). Reserpine treatment shows after 48 hours a marked decrease in the intensity of both fluorescences.

Immunohistochemical observations

Specificity. The preabsorption of the antisera with the homologous antigens resulted in abolishment of the reaction.

Immunoreactivity related to Substance P

In Fig. 3A the occurrence of a-SP immunoreactive cell bodies and fibres in the CNS of P. corneus is depicted. Positive perikarya and fibres were observed in all ganglia. The buccal ganglia are characterized by a pair of large symmetrical neurons (60-70 µm) localized dorsally in a rostral position. Other two pairs slightly smaller in size are localized more posteriorly: the first is in a ventromedial location (about 40 µm in

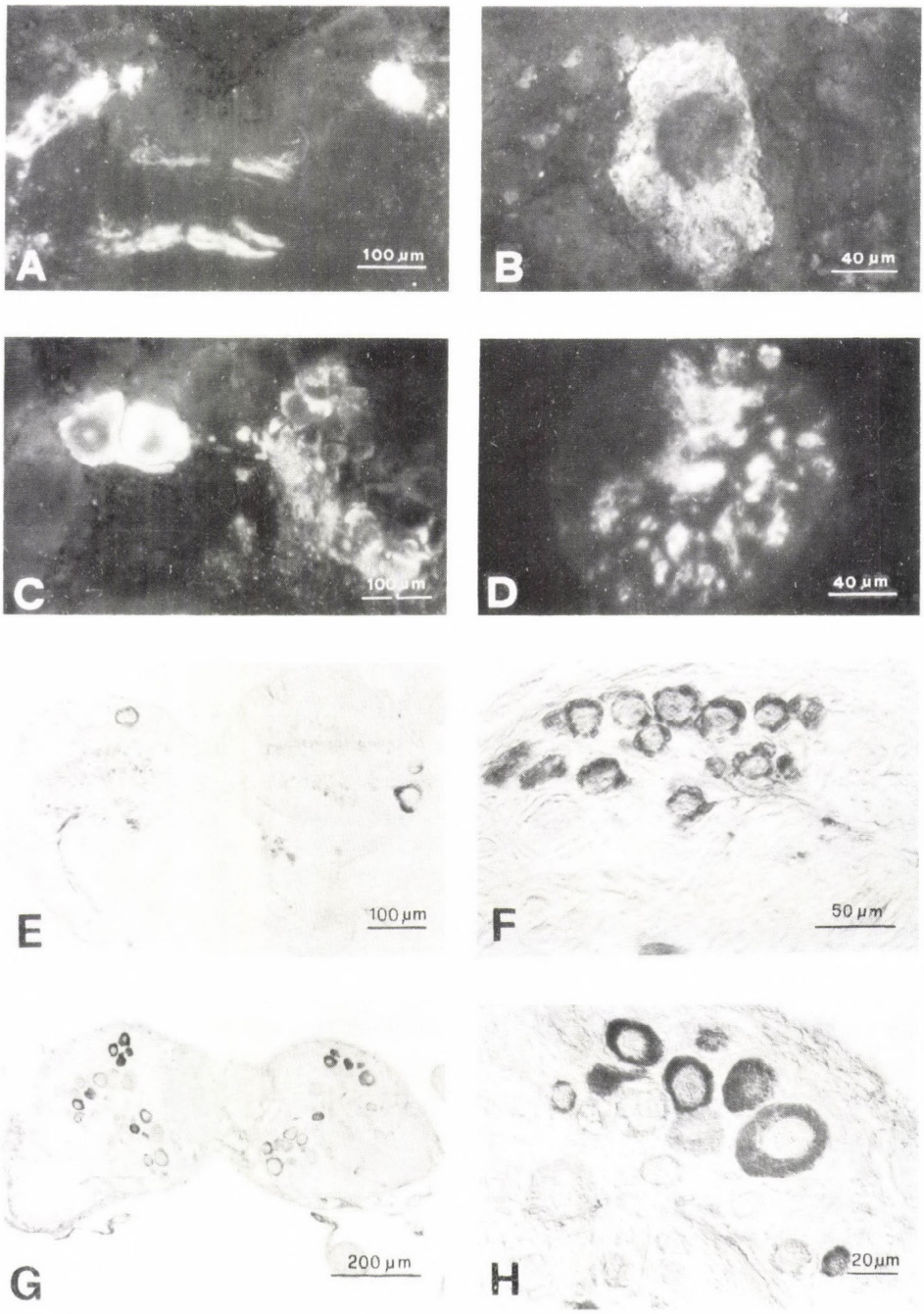

Fig. 2

size); the second pair is in dorsolateral position (45 µm in size). Both pairs send axons into the buccal commissure. There are some further scattered groups of small cells. Immunopositive fibres are seen in the neuropils where they assume a beaded appearance and in all buccal nerves and the cerebrobuccal connectives (Fig. 2E). In the cerebral ganglia no large positive neurons are observed. A peculiarity is the presence of strongly stained symmetrical groups of cells (6-8) small to medium in size (10-45 µm). Two of these are localized dorsally, near the cerebropedal connective and in anteromedial position. Some other groups of cells are found on the ventral surface. There are also several scattered cells medium in size (35-40 µm). The cerebral neuropils are rich in immunopositive fibres and varicosities. From here bundles of processes cross the cerebral commissure and run to the cerebropedal and cerebropleural connectives. Positive fibres are found only in the medium labial nerves. In the right pleural ganglion an immunoreactive neuron is observed with a multipolar appearance (about 35 µm in size). Instead positive fibres run in both pleural ganglia (Fig. 3A). The most numerous and characteristic cluster of cells of variable size (35-80 µm) is found in the pedal ganglia (Fig. 2F). The neurons (40-50 cells) lay in symmetrical location on the ventral side around the exit of the pedal inferior nerve and send their axons to the centre of the neuropils. From there bundles of immunoreactive fibres run to all nerves and connectives. There are at least two other small groups of cells (5-6 in number, 20-25 µm in size). Some neurons

←

Fig. 2. A-D: SPG technique. E-H: Immunocytochemistry. A) Transverse section of the cerebral ganglia showing yellow fluorescent symmetrical clusters of cells and bundles of fibres crossing the intercommissure. B) Giant Dopaminergic Cell on the ventral side of cerebral ganglia showing also yellow fluorescence at the excitation wavelength for 5-HT. C) Two large blue-green neurons and several small yellow cells in a pedal ganglion. D) Dopaminergic fibres in a transverse section of a pedal nerve. E) SP-like immunoreactive neurosomata and fibres with a beaded appearance in the buccal ganglia. F) SP-like immunoreactive cell cluster in the left pedal ganglion. G) Frontal section of the cerebral ganglia showing symmetrical FM-like immunoreactive cells. H) Different degree of reactivity to a-FM in cell bodies of cerebral ganglia.

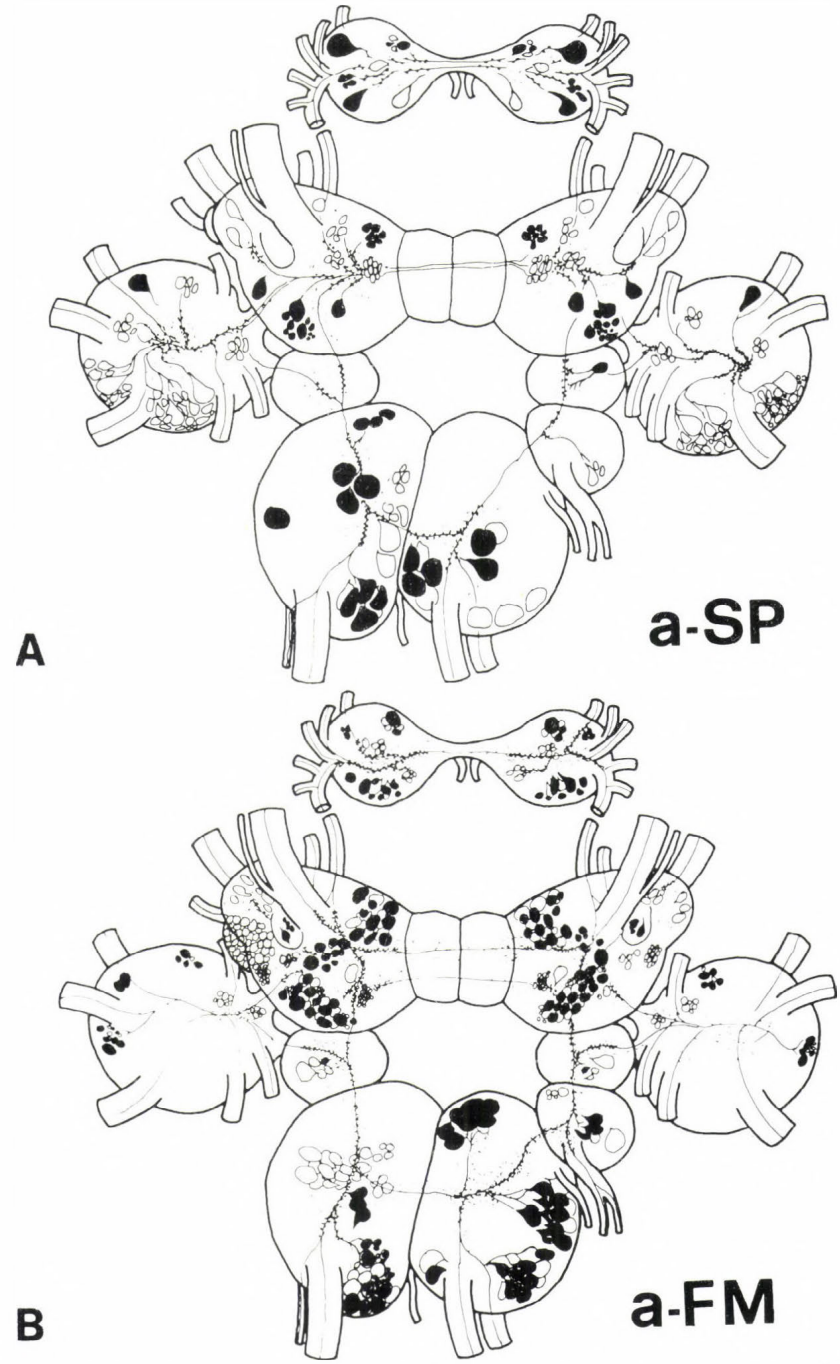

Fig. 3. Maps of neurons and fibres showing SP- and FM-like immunoreactivity. Dorsal cells are indicated by solid circles and ventral cells by open circles.

of medium size (35-45 µm) are also scattered. In the visceroparietal ganglia several positive neurons of considerable size (80-100 µm) are found in various locations. The most characteristic ones are grouped along their adjacent surface. In the right parietal ganglion only 2-3 cells are present near the exit of the pallial nerve. Numerous positive fibres are seen in the visceroparietal neuropils where they assume a rough apearance and from here they turn to all nerves and connectives (Fig. 3A). The comparison on consecutive sections of SP-like immunoreactivity with AB/AY staining has shown no correspondence between this peptide and classical neurosecretion.

Immunoreactivity related to FMRFamide

In Fig. 3B the distribution of cell bodies and fibres identified by anti-FM serum is shown. Positive nerve cells and fibres are seen in all ganglia with a predominance of perikarya in the cerebral and visceroparietal ones. Two groups of 5-10 immunoreactive cells (18-46 µm in size) are present in the buccal ganglia. Some other cells are located on the ventral side in a rostral position (4-6 cells, 30-40 µm in size). Numerous positive fibres cross the neuropils and reach all the buccal nerves and the buccocerebral connectives. Many positive granules are present in the neuropils. The majority of immunoreactive cells are found in the cerebral ganglia symmetrically located on the dorsal surface (Fig. 2G-H). Forty to fifty perikarya (30-40 µm in size) are arranged between the exit of the tentacle nerves and the cerebropedal connectives. Other neurons are located in anterodorsal position. Groups of small cells (10-15 µm) are observed ventrally. The most characteristic cluster of 40-50 cells (20-25 µm) is asymmetrically placed in the ventral lobe of the left ganglion. These neurons innervate the penial region. In each lateral lobe there is a positive bipolar cell (40 µm) and some other small ones. Bundles of positive fibres with a rough appearance cross the neuropils and turn towards the cerebropedal and cerebropleural connectives. Positive fibres are seen in all the cerebral nerves. Only a small group of 4-6 positive cells (25-40 µm) is found in each

pleural ganglion. Few positive cells are found in the pedal ganglia despite the numerous fibres. Two to three small groups of cells are ventrally located near the origin of the nerves and in the medial region (5-8 cells, 25-35 μm). Positive rough fibres run into the neuropils and all the nerves. In the right parietal ganglion two small clusters (3-5 cells each; 15-40 μm) are located near the origin of the right pallial nerve. A giant neuron (95-100 μm) is also weakly positive. In the left parietal ganglion there is a conspicuous immunopositive cluster caudally located near the origin of the left external pallial nerve (30-35 cells, 25-90 μm). Another group lies ventrally (15-20 cells, 40-60 μm). Some other small groups of 3-4 cells (30-40 μm) are ventrally located in a medial position. In the visceral ganglion a conspicuous group of 20-30 cells (50-90 μm) is caudally located near the origin of the intestinal nerve. Another group of cells is situated dorsally in rostral position. A cluster of numerous monopolar neurons with the axons turning towards the middle of the ganglion is situated in a ventrolateral location. Bundles of positive fibres cross the visceroparietal ganglia and many fibres run in all the visceroparietal nerves. Numerous positive granules and rough fibres are present in the neuropils (Fig. 3B). Using the AB/AY staining comparison, an AY positivity was found for some FM-like immunoreactive neurons in the visceroparietal ganglia.

Adenylate cyclase activity

In Fig. 4 the dose-response relationships of FM and SP to adenylate cyclase activities in CGC, PGC and VPC homogenates are depicted. As shown, FM up to 10^{-4} M is quite ineffective on basal enzyme activity in all the tested ganglia. An inhibitory effect was always observed only at the maximal dose used (10^{-3} M). The basal adenylate cyclase activities, which are 67.1±7.1 in CGC, 58.5±5.0 in PGC and 62.2±7.2 pmol cAMP/mg prot/5 min in VPC, are reduced to 65%, 80% and 74%, respectively, in the presence of 10^{-3} M FM. As also shown in the same figure, SP, when present in lower doses, slightly increases in all the preparations, the enzyme activity inducing a maximal

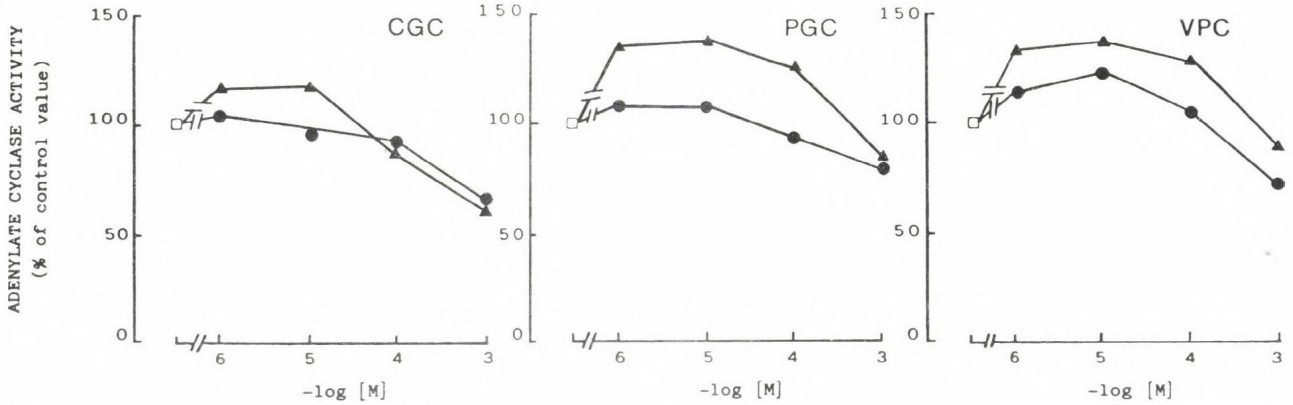

Fig. 4. Dose-response curves of the effects of FM (●—●) and SP (▲—▲) on adenylate cyclase activity in homogenates of CGC, PGC and VPC. □: Control values. Basal enzyme activities: 67.1±7.1 pmol cAMP/mg protein/5 min in CGC; 58.5±5.0 pmol cAMP/mg protein/5 min in PGC; 62.2±7.2 pmol cAMP/mg protein/5 min in VPC. Data are the mean findings of three experiments.

stimulation of 119% in CGC and of 137% in both PGC and VPC. The highest dose (10^{-3} M) tested always inhibits the adenylate cyclase activity which is reduced to 64% in CGC, 83% in PGC and 92% in VPC.

In Fig. 5 the effects of different concentrations of FM and SP on the 5-HT-stimulated adenylate cyclase activity are depicted. As shown, 10^{-4} M 5-HT greatly stimulates the enzyme activity in all preparations (252% in CGC, 287% in PGC and 213% in VPC). FM at 10^{-6} to 10^{-3} M inhibits the amine effect in all ganglia. At the maximal dose it reduces 5-HT stimulation to almost 50%.

In the presence of 5-HT, SP induces a bimodal regulation of adenylate cyclase activity: in all ganglia, a slight potentiation of 5-HT effect is observed when the neuropeptide is 10^{-6} M, while an inhibitory action is observed at higher doses. 10^{-3} M SP reduces 5-HT-stimulated adenylate cyclase activity to 25-50%.

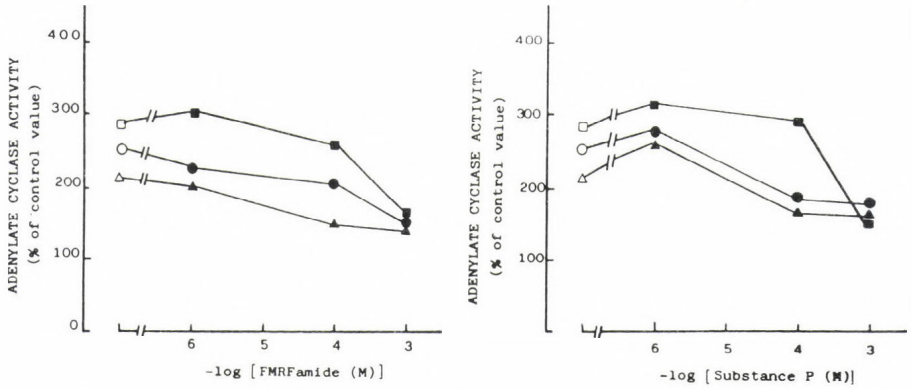

Fig. 5. Effect of FM and SP on 5-HT-stimulated adenylate cyclase activity in homogenates of CGC, PGC and VPC. ●—● CGC; ○ 5-HT alone; ■—■ PGC; □ 5-HT alone; ▲—▲ VPC; △ 5-HT alone. Basal enzyme activities are as reported in Fig. 4. 5-HT is 0.1 mM. Data are mean findings of three experiments.

DISCUSSION

Marsden and Kerkut (1970) were the first to investigate the occurrence of monoamines in the CNS of P. corneus using the Falck-Hillarp histofluorescence procedure. They showed by

spectrofluorimetric assays that 5-HT and, among the catecholamines, only DA were present in neurons and fibres. By using the glyoxylic acid method we were able to make the same observations but also to detect neurons previously not described due to the higher sensitivity of this method.

The occurrence and wide distribution of SP- and FM-like immunoreactivity in the ganglia of P. corneus are in agreement with results obtained in different molluscs (Schot et al. 1981, Schot and Boer 1982) and other invertebrates (Kuhlman et al. 1985, Dhainaut-Courtois et al. 1985). The specificity of the immunochemical reactions was provided by using non-immune sera and absorption controls. Furthermore, we obtained a comparable distribution of immunoreactive material by either indirect immunofluorescence or the DAB reaction. Nevertheless, we must be cautious in interpreting these results since the exact identity of the active peptide can be revealed only by further biochemical analysis. For instance, it has been shown that the existence of many closely related analogs of FM and the members of this large family probably share antigenic determinants. We cannot state here if FM- or SP-like peptides in P. corneus are of a neuroendocrine nature or are more conventional neuromediators. In the case of FM-like immunoreactivity we found a partial correspondence with classical neurosecretory staining but, on the other hand, we observed the same immunopositivity in cell bodies and processes of identified motoneurons that command the tentacle movements.

The possibility that molecular events underlying FM and SP effects could be mediated intracellularly by membrane enzymes has been referred to by contradictory results. Weiss and Drummond (1985) reported the presence of an adenylate cyclase activity stimulated by FM in the gill of Aplysia. Colombaioni et al. (1985) demonstrated that in isolated neurons of Helix, FM induces a 3-fold stimulation of the enzyme activity. On the contrary, Ocorr and Byrne (1985) reported that in Aplysia sensory neurons FM has little or no effect on cyclic AMP levels. Brezina et al. (1987b) suggested that this peptide activates a GTP-binding protein, but they excluded the involvement of the currently known second messengers. Concerning SP, Duffy

and Powell (1975) reported that this peptide stimulates the adenylate cyclase activity in the rat and human brain. Volle and Patterson (1982) reported an increase of cyclic AMP levels in rat sympathetic ganglia exposed to SP. On the other hand, it has been reported that in several tissues SP exerts its effects without influencing the cyclic AMP system (Lee et al. 1983) and it has been assumed that SP might influence the phosphatidylinositol turnover (Tanaka et al. 1986).

In the CNS of P. corneus the effects of the neuropeptides FM and SP on the adenylate cyclase activity are less evident and more complex with respect to the obvious stimulation observed in the presence of 5-HT. 5-HT and DA effects on the adenylate cyclase activity in CGC, PGC and VPC homogenates have been extensively described in another paper (Sonetti et al. 1987) in which we reported that only 5-HT stimulates the enzyme activity markedly while DA is completely ineffective in all the ganglia, in spite of the fact that it stimulates cyclic AMP synthesis in the whole ganglia. Thus we assumed that DA, possibly by releasing 5-HT, indirectly influences the nucleotide concentration. In lower doses, FM seems to be ineffective on the adenylate cyclase activity in CGC and PGC, while a slight stimulation of enzyme activity is observed in the VPC when FM is 10^{-5} M (125%). At a very high neuropeptide concentration there is a considerable inhibitory effect in all the preparations. SP, on the contrary, elicits a dual regulation of the adenylate cyclase activity in all ganglia. As in the case of FM, the inhibitory effect of SP observed at 10^{-3} M might actually be due to the excessive dose used. If so, SP could exert only a slight stimulation as reported in other systems (Volle and Patterson 1982). Alternatively, we believe that receptors with different affinities for SP lead to stimulation or inhibition of the adenylate cyclase activity, depending on the type of G protein preferentially activated. On the other hand, in a different preparation, we were surprised to find the neuropeptide met-enkephalinamide to inhibit cyclic AMP synthesis when present alone, but to greatly enhance nucleotide levels when tested in combination with prostaglandin E_2 (Borasio et al. 1986). Since 5-HT is very effective in stimulating the adenylate cyclase ac-

tivity in the CNS of P. corneus, we tested the effects of FM and SP on the 5-HT-stimulated adenylate cyclase activity to verify the possible interactions at the level of the cyclic AMP system. The results indicate an inhibitory effect for FM and confirm the dual regulation by SP of the enzyme activity.

On the basis of the evidence presented so far, we assume that in the CNS of P. corneus, in addition to amines whose role of neuromediators in the invertebrate nervous system is well known, also FM and SP might possibly be modulatory substances as evidenced in other molluscan species. Our results seem to indicate that in selected ganglia of P. corneus, FM and SP influence the cyclic AMP system, especially when the synthesizing enzyme is activated by 5-HT. On the other hand, in the same preparations, DA is not only ineffective on the basal adenylate cyclase activity but does not influence the 5-HT-stimulated enzyme activity either (Sonetti et al. 1987). Not surprisingly, our report shows that in the CNS of P. corneus, FM counteracts the stimulatory effect of 5-HT on the cyclic AMP system. In fact, it has been reported that in another system the same function is modulated by 5-HT and FM in the opposite directions (Belardetti et al. 1987, Brezina et al. 1987b).

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DISCUSSION

ERDÉLYI, L.: I have two short questions. First, have you identified two different peptides in the same neuron? Second, have you tried to use capsaicin to deplete SP or other peptides?

SONETTI, D.: 1. This time I found one case of possible coexistence of two neuropeptides in the same neuron. The cell cluster on the ventral surface of the left cerebral ganglion innervating the penial contractor muscles as well as some neurons in the visceroparietal ganglia react positively to both anti-FMRFamide and anti-NPY. I controlled this co-localization on consecutive sections of the same cells immunostained differentially. These results will be published at length in a forthcoming paper.

2. I did not try capsaicin to deplete SP or other peptides and I do not know if it has been utilized in molluscs. So it seems to me a good suggestion to try it.

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BURSICON IN THE PROTHORACIC REGION OF CRICKETS

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Bursicon is a neurohormone which initiates hardening and darkening of the cuticle after emergence in flies (Cottrell 1962, Fraenkel and Hsiao 1962) and in other insects (Fraenkel and Hsiao 1965, Truman 1973, Abboud et al. 1983). It is a protein which is degraded by proteases and survives heat treatment of 80°C (Mills and Lake 1966, Reynolds 1977, Taghert and Truman 1982a; for review see Seligman 1980). Bursicon is produced by four neurosecretory cells in each of the unfused abdominal ganglia in Manduca sexta and is released through the abdominal neurohaemal perivisceral organs (PVO) in Manduca and Tenebrio molitor (Truman 1973, Grillot et al. 1976, Taghert and Truman 1982b). However, in Manduca, the presence of bursicon can also be detected in homogenates of the brain, suboesophageal and thoracic ganglia and in the locust in the thoracic ganglia and corpora cardiaca (Vincent 1972).

In Orthoptera, a cluster of somata in a distinct posterolateral position on either side of the prothoracic ganglion (PTG) are the only cells projecting into the PVO of the neck region, the median nerve with its branchings in the left and right transverse nerve (MN-TN complex) between the PTG and the suboesophageal ganglion (SOG) (see Figs 1 and 2). These cells have been found to show FMRF- and BPP-immunoreactivity in locusts (Myers and Evans 1985a,b). Bearing in mind that in Manduca sexta four cells in each unfused abdominal ganglion contain bursicon and release it via abdominal PVOs, we aimed to investigate the neurons of the posterolateral clusters in the PTG for bursicon activity.

We found that in adult crickets the PTG and the MN-TN complex between the PTG and SOG contain tanning activity but not the cells of the two posterolateral cell clusters in the PTG. Instead, it was revealed that peripheral neurons in the prothorax, which project to the MN and other prothoracic nerves, contain bursicon-like activity. They may be one source of bursicon-like activity of the PTG, the MN-TN complex and other prothoracic nerves.

METHODS

Adult crickets (Gryllus bimaculatus) of an age of at least 14 days after the imaginal moult were taken from a permanent laboratory culture or were kindly supplied by the laboratory of F. Huber, Max-Planck-Institut für Verhaltensphysiologie, Seewiesen. Tissues of the ventral nervous system were dissected under saline and immediately transferred into a drop of icecold saline on a slide. Tissues of 15-20 animals were collected and stored at -30°C until required.

As a standard, 10 prothoracic ganglia, or MN-TN complexes were homogenized in 100 µl saline without glucose (Honegger and Schürmann 1975) at 4°C, i.e. approximately 30-fold the wet weight of the PTG-tissue. Of the paired structures such as nerves or peripheral cell clusters, 10 pairs of the tissues were homogenized in 100 µl saline. The homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatants were used directly for injection or stored at -30°C.

Digestion with protease K was carried out with homogenates of the prothoracic ganglion and the MN-TN complex of standard concentration. The supernatants were incubated with 4.4 units of protease K (Sigma) at pH 7.0 and 37°C for 3 h. Enzyme incubations were terminated by heating to 85°C for 10 min.

All homogenates were tested for bursicon activity with the aid of the bioassay on ligated blowflies (Fraenkel and Hsiao 1965, Abboud et al. 1983; for review see Seligman 1980). Newly emerged blowflies were neckligated and injected with 5 µl of the test solution if they remained untanned 1 h after ligation.

The injected flies were kept for three hours at room temperature and then immersed in 70% ethanol. The sclerotization was quantified as described by Fraenkel and Hsiao (1965). Their scoring system was:

3	+++	fully tanned, cuticle appears uniformly dark;
2	++	the larger part of the cuticle is dark;
1	+	tanning is limited to small patches;
0.5	+/-	some small indication of tanning;
0	-	entirely untanned.

We scored thorax and abdomen separately, each with a score of maximally 3 points. The results for both thorax and abdomen were added to give a maximum score of 6 for one fly. Usually, 20 flies were injected with one test solution. The scores of the single flies were added and divided by the number of injected animals. As a control 20 flies were injected with saline in each test series. Tests were discarded if the mean score of the saline control was above 0.54 points.

The PNC and their projections were revealed by using cobalt chloride and silver intensification as described in Honegger et al. (1985).

RESULTS

In order to test our hypothesis that the cells of the posterolateral cell cluster in the PTG (PL-cells) of crickets contain bursicon we first tested homogenates of both the PTG and the MN-TN complex in the ligated fly assay (see Methods).

Bursicon-like activity was detected in the homogenates of both tissues (Table 1; 1 and 2). Homogenates of both the MN-TN complex and the PTG incubated with protease K lost their tanning activity (Table 1; 3) indicating that the activity is associated with a protein. As a further step, triangular segments of either side of the posterior PTG which contain the PL-cells were isolated, homgoenized and tested. Homogenates of a segment of either side of the anterior PTG between nerve 1 and nerve 3 which does not contain any branchings of the PL-cells (Fig. 1) served as a control. Since the somata of the PL-cell cluster

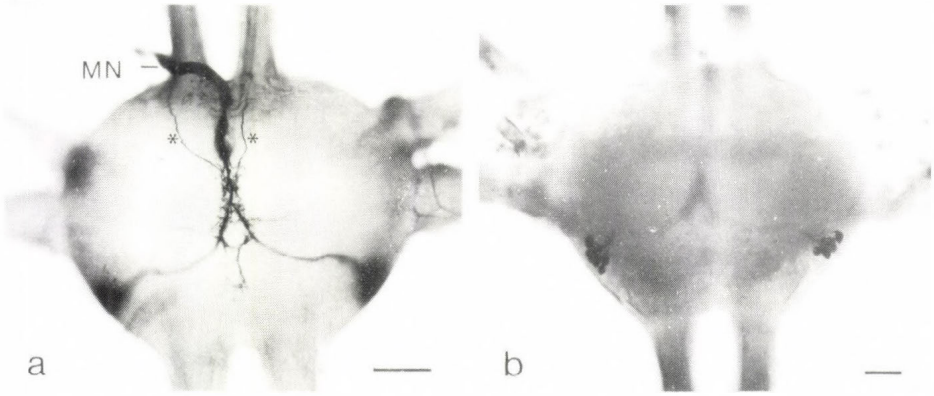

Fig. 1. Cobalt stains of the two posterolateral cell clusters in the prothoracic ganglion of Gryllus bimaculatus. Backfills from median nerve. a: Branching pattern of the cells in the prothoracic ganglkon, dorsal view, larva, VIIth stage; b: ventral view reveals the cell bodies on either side of the ganglion, adult cricket; *: axons of two neck muscle motoneurons (Honegger et al. 1985); scale 100 µm

Table 1. Tanning response of ligated flies after injection of homogenates of different tissues of the prothoracic nervous system of adult crickets

Homogenate	$\bar{x}$	±	s	n tests
1. PTG	3.45		0.67	25
2. MN-TN	3.60		0.84	10
3. Protease K, MN-TN and PTG*	0.1	and	0.3	1 each
4. Ant. segments of PTG	1.14		0.58	3
5. Post. segments of PTG	0.26		0.18	4
6. N1/PTG x 2.8	4.1			1
7. N3/PTG	2.3		0.97	6
8. Branches G/H of N3/PTG	0.36		0.36	3
9. N4/PTG	0.20		0.23	3
10. N5/PTG	0.24		0.05	6
11. N8/SOG x 1.6	2.56			1
12. PNC	3.33		0.72	6

$\bar{x}$: mean of the means of n separate tests with standard deviations s of the means. Results shown in the Table include 44 Ringer controls with mean tanning score of 0.21 and standard deviation of 0.15; maximum Ringer score of one test was 0.52. *: One homogenate of MN-TN and PTG, respectively, was incubated with protease K (see Methods).

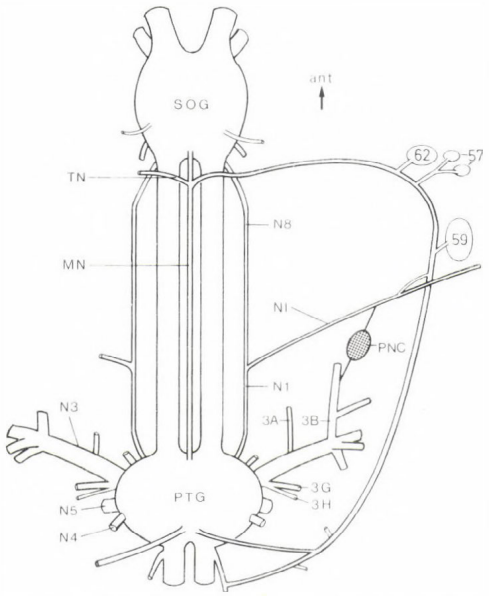

Fig. 2. Innervation scheme of the neck region between the suboesophageal ganglion (SOG) and the prothoracic ganglion (PTG) of crickets; modified from Honegger et al. (1985). All major nerves show innervate muscles and sensory structures in the neck and prothorax. Note that the median nerve (MN) branches in 2 transverse nerves (TN) which innervate neck muscles (M62, M57) and form connections to other peripheral nerves of the PTG. The shaded structure between NI and N3B contains three peripheral neurosecretory cells on either side of the PTG (cf. Fig. 3)

have a distinct and unambiguous position (Fig. 1), we can assume that the two posterior segments contained all PL-cells. Bursicon-like activity could be detected only in the homogenates of the anterior PTG segments (Table 1; 4 and 5). A test where homogenates with a double concentration of the PTG segments were injected showed an even larger difference in the score between anterior and posterior (3.43 and 0.5, respectively) than that shown in Table 1 (4 and 5).

Since we could not stain any neurons in the anterior PTG which project into the median nerve we considered peripheral neurosecretory (neurohaemal) cells (PNC) in the neck region as a possible source of bursicon-like activity in the PTG and the prothoracic PVO. Three cell bodies on either side of the PTG

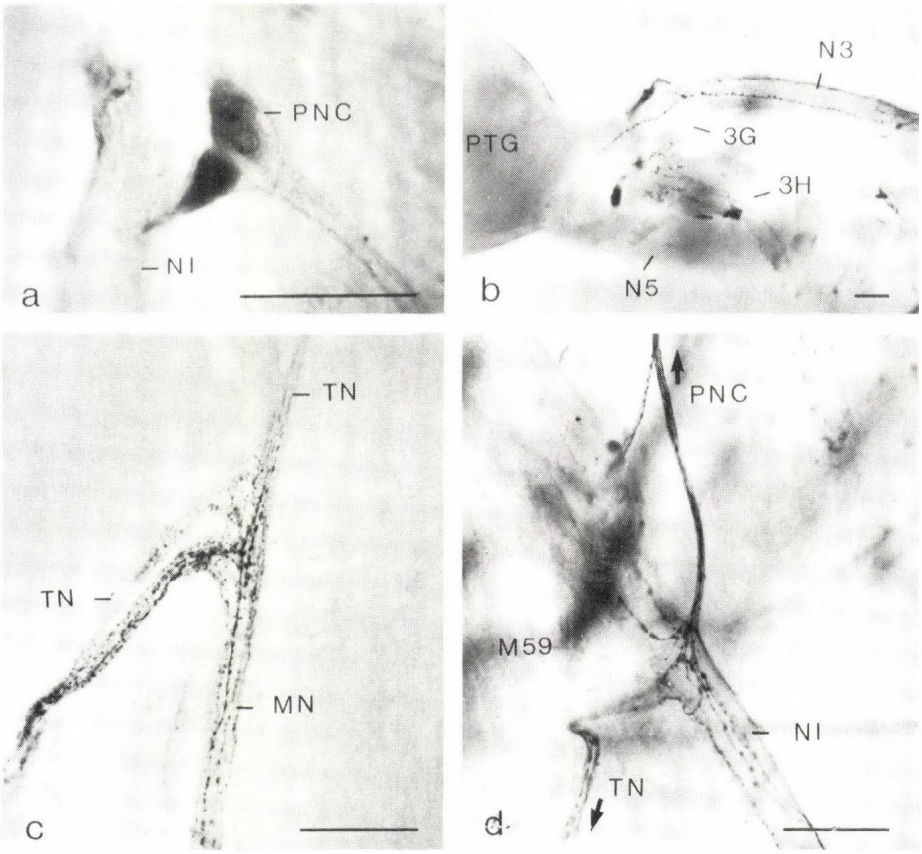

Fig. 3. Peripheral neurosecretory cells (PNC) and their projections onto prothoracic nerves in Gryllus bimaculatus. a: Cobalt backfill of the ipsilateral transverse nerve. Three cell bodies (PNC) are closely attached to nerve I (NI). The nerve projecting to the lower right corner is the connection of the PNC to nerve N3B (cf. Fig. 2). b: Cobalt backfill from the loop between muscles 57 and 59 stained the PNC network on the proximal part of nerve 3 and its branches G and H; prothoracic ganglion (PTG) and nerve 5 (N5) as markers. c: Cobalt stain from the PNC to the median nerve (MN) and transverse nerves (TN). d: Cobalt backfill from TN (arrow at bottom). Fine branches of the PNC can be seen on NI and the fine nerves which connect with the PNC (arrow at top). In this preparation the PNCs are not located directly at NI as in a. Scales 100 µm

lie between nerve Ni and nerve N3B (Fig. 2) in a common sheath and send projections to all anterior nerves of the PTG and the common nerves of the SOG and PTG, i.e. N8/SOG, N1/PTG, NI,

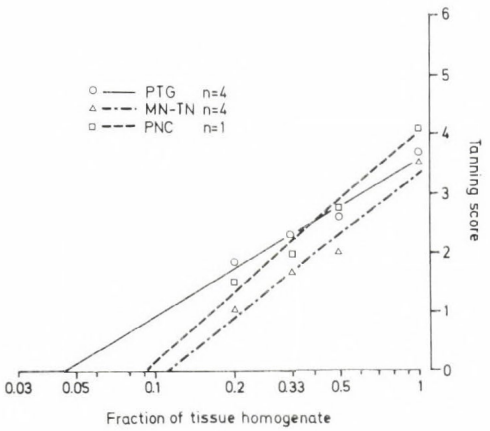

Fig. 4. Mean tanning response of ligated flies to various dilutions of homogenates of the prothoracic ganglion (PTG), the median-transverse-nerve-complex (MN-TN) and the peripheral neurosecretory cells (PNC). "1" on the abscissa represents the homogenate of one PTG, one MN-TN or two PNC clusters in 10 µl saline respectively (see Methods). n: Number of separate tests constituting the points of each curve. Curves are linear on the logarithmic scale with slopes and correlation coefficients of 1.14, 0.83 for PTG; 1.5, 0.78 for MN-TN; 1.64, 0.99 for PNC

N3/PTG, the MN-TN complex and N3 H,G/PTG in 1 of 10 preparations (cf. Fig. 2). All these nerves showed a fine network of varicose fibres (Fig. 3). The fibres appear to lie in the connective tissue sheath of all nerves except the MN-TN complex where dense fine branchings can be distinguished throughout the whole nerve.

To test for bursicon activity, the PNC and a proximal segment of a distinct size of all nerves on which these PNC project were isolated and homogenized. Table 1 (6, 7, 11, 12) shows that bursicon-like activity can be detected in all homogenates of the nerves which carry the projections of the PNC. Since the PNC-net seems to spread over the surface of the nerves, and N1/PTG and N8/SOG have a much smaller diameter than N3/PTG, a corresponding larger amount of material from the two smaller nerves was homogenized to compensate for their smaller diameter. Table 1 indicates how much more material of N1/PTG and N8/SOG was taken, assuming that the PNC nerve net has a similar density in all nerves. The homogenates of the posteroproximal

branches of N3, branches G and H (see Fig. 2) have a negative tanning score. This is consistent with the result that PNC-projections could be identified in only 1 of 10 backfills where the proximal part of N3 carried projections (Fig. 3). Bursicon-like activity cannot be detected in homogenates of the posterior nerves of the PTG which do not contain the net-like projections of the prothoracic PNC, i.e. N4 and N5.

This result indicates that the three prothoracic PNC on either side of the PTG must be the source of the bursicon-like activity in the anterior prothoracic nerves, the anterior prothoracic PVO and presumably in the anterior PTG. Serial dilution of the homogenates of the PTG, the MN-TN complex and the PNC (Fig. 4) indicates a logarithmic dose-response relationship for all three tissues. The similar slope of the three curves in Fig. 4 indicates moreover that the tanning material of the three tissues is identical.

DISCUSSION

This study shows that in adult crickets a protein which has the effect of bursicon in the ligated fly assay is contained in peripheral neurons and their projections. However, there is no evidence for the presence of bursicon-like activity in the haemolymph of adult crickets, a result supported by earlier tests with the haemolymph of adult flies (Cottrell 1962, Fraenkel and Hsiao 1965) and of pharate adults Manduca sexta and Tenebrio molitor (Reynolds et al. 1979, Abboud et al., 1983). The most plausible explanation for this discrepancy seems to be that a low bursicon concentration cannot be detected by the bioassay used. Our dose-response curve (Fig. 4) shows that about 0.3% of the wet weight of one cricket PTG is the limit of detection in our assay.

If bursicon is present in adult insects, it may have additional functions to the initiation of tanning. Mills and Whitehead (1970) showed that bursicon may be associated with diuretic activity during ecdysis. Bursicon is not a cardioactive

factor (Tublitz and Truman 1985). To our knowledge, other functions of bursicon have not been tested in adult insects.

Peripheral neurosecretory cells have been shown in a variety of insects (for review see Raabe 1982). We found that in crickets one set of peripheral neurons, the PNC of the prothoracic nervous system and their processes, are associated with bursicon-like activity. The positive bursicon response of the anterior part of the PTG cannot be correlated with the occurrence of PNC-fibres. None of our cobalt preparations showed any indication of PNC-fibres on the PTG. However, the PNC-projections are small in diameter and we assume that not all projections might have stained to their full extent. As an alternative, bursicon-containing cells which we could not identify yet may be located in the anterior PTG.

It has been shown that the neural sheath of the blowfly, locust and cockroach also contain serotonergic fibres (Nässel and Elekes 1985, Bräunig 1987, Davis 1987) and in lobster serotonin-proctolin fibres (Beltz and Kravitz 1987). Thus a large part of the nervous system in invertebrates seems to constitute a general neurohaemal region for the release of a variety of neurosecretory material into the haemolymph.

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DISCUSSION

van der WILT, G.: Did you stain the peripherally located neurons by backfilling the median nerve?

HONEGGER, H.W.: We could not stain the peripheral cells from the median nerve but only from the transverse nerves. However, as I showed, we could stain the PNC fibres in the median nerve from the peripheral cell bodies. Please keep in mind that these fibers are very fine and the distance to the median nerve is very long.

van der WILT, G.: Do you think that these neurons are amenable to electrophysiological studies?

HONEGGER, H.W.: We did some preliminary extracellular recordings from the nerves to which these cells project. The cells are electrically active and fire with long action potentials. I am doubtful whether it would be possible to record intracellularly from these cells. They are not supported by a larger mass of connective tissue and are very small.

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THE ANTENNAL HEART OF THE AMERICAN COCKROACH. A NEUROHORMONAL RELEASE SITE CONTAINING OCTOPAMINE

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ABSTRACT - The antennal heart in the head of *Periplaneta americana* is not only a pump for haemolymph supply of the antennae but also a neurohaemal organ. Within the walls of its ampullae numerous neurosecretory axons terminate originating from DUM-neurones in the suboesophageal ganglion (retrograde cobalt iontophoresis). A very high amount of octopamine could be detected in the antennal heart by biochemical methods (dansylation/TLC and radioenzymatic assay/HPLC). The bulk of this substance is concentrated in the walls of the ampullae suggesting a release from the neurohaemal area into the haemolymph pumped immediately to the antennae. A possible modulatory role of octopamine for the antennal sensory system is discussed.

Close spatial relationships between circulatory and hormonal organs can often be observed. The functional significance of such an association is easy to understand, as it provides ideal release sites for a rapid and homogeneous distribution of hormones in the body. This is especially true for animals with an open circulatory system, like insects. Their most prominent neuroendocrine organs, the corpora cardiaca, for example, are located at the anterior end of the tubular dorsal vessel. Beside this large circulatory motor further small pulsatile organs exist in insects effecting haemolymph circulation in longer body appendages as antennae, legs or wings (review: Miller 1985). In such an accessory circulatory organ a further association with the neuroendocrine system has been observed, namely in the antennal heart of the cockroach *Periplaneta americana*. This organ was first described by Pawlowa in 1895 and has received only little interest since then. It is obviously a basic structure in insects and deserves special attention by

neurobiologists, as it represents a relatively clearly arranged nerve-muscle system. Furthermore, the antennal heart may be of particular importance for the function of the antennal sensory apparatus, so far completely ignored by sensory physiologists. For these reasons we have focused our efforts on this minute circulatory organ; our research includes a reinvestigation of the functional morphology (Pass 1985), a study of its innervation (Pass et al. 1987a), electrophysiological and pharmacological experiments (Hertel et al. 1985), and biochemical investigations (Pass et al. 1987b).

The antennal heart lies directly under the frontal cuticle and consists of two ampullae joined by a transverse muscle (Fig. 1). Blood vessels from these ampullae extend into the antennae and open distally. The haemolymph then flows in the haemocoel of the antennae from the tip back to the head. One can, therefore, speak of an antennal circulation, in the true sense of the word. The proximal portions of the antennal vessels in the head are curled and form glomerula-like structures. The electron-microscopic investigation revealed that the wall of this vessel portion consists of a transport epithelium and we believe that these cells are in some way involved in ion- and/or osmoregulation of the haemolymph which is pumped into the antennae (Pass 1985). The transverse muscle inserts on the inner walls of the ampullae and one can well imagine that upon contraction the lumina of both ampullae expand simultaneously. Haemolymph then flows through small ostia into the ampullae. After relaxation of this muscle the elasticity of the walls of the ampullae returns them to their original shape.

The function of the antennal heart as a neurohaemal organ has been earlier recognized by Beattie (1976) by the numerous neurosecretory terminals which lie within the ampullar walls (Fig. 2a, b). This wall has a spongy character, i.e., it consists of only very thin epithelial cells and voluminous extracellular cavities. The neurosecretory terminals stem from axons which run along the dilator muscle and extend into these spaces up to the inner basal lamina. They are without a glial sheath and densely packed with neurosecretory granules. Structures characteristic of the release of neurosecretory material such as omega profiles or microvesicles can be observed. From the morphological situation we must assume that the secretions released there enter the ampullar lumen and are carried by haemolymph immediately into the antenna. They, therefore, obviously have nothing to do with the control of the antennal heart rhythm.

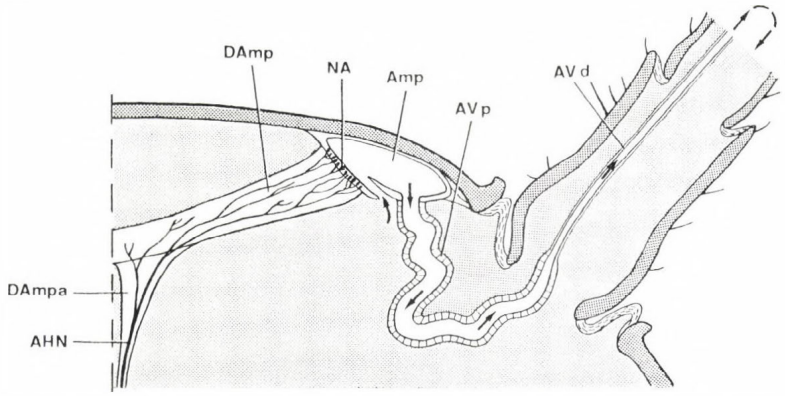
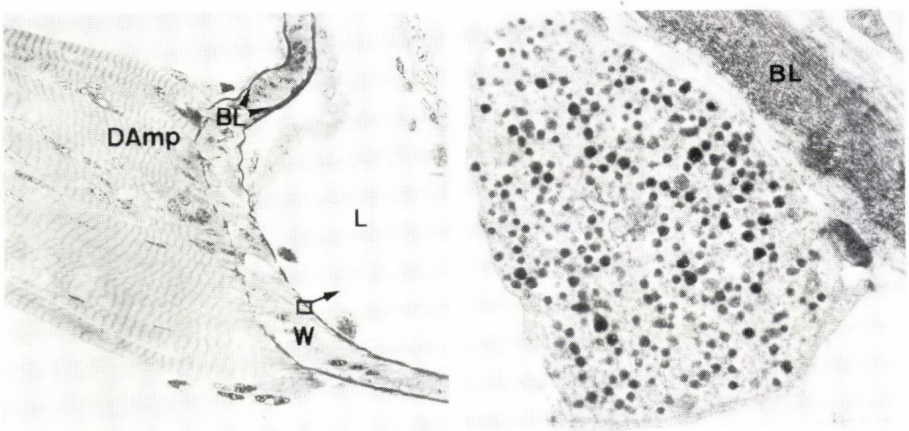


Fig. 1. Schematic section through the right half of the antennal heart of *Periplaneta americana*. *Amp* ampulla, *AHN* antennal heart nerve, *AVd* distal portion of antennal vessel, *AVp* proximal portion of antennal vessel with transport epithelial wall, *DAmp* dilator of ampullae, *DAmpa* accessory dilator of ampullae, *NA* neurohaemal area. Arrows indicate the haemolyph flow.



a b

Fig. 2. Sections through the antennal heart of *Periplaneta americana*. **a.** A right ampulla with the inserting dilator muscle *(DAmp)*, *L* lumen of ampulla, *W* ampulla wall with thick basal laminae *(BL)*. The frame indicates the detail magnified in **b** showing a neurosecretory terminal in the ampulla wall. Note the large granules of various electron-density and the numerous clear microvesicles. *a* x480; *b* x22000

A neuroanatomical study with axonal cobalt iontophoresis revealed that these terminals probably stem from axons of the antennal heart nerve (Pass et al. 1987a). This paired nerve extends from the large dilator muscle under the brain back to the retrocerebral glands. The axons run through the corpora cardiaca and in the circumoesophageal connectives to the suboesophageal ganglion where the perikarya are located. Important in our respect is only one special neurone type in the dorsal anterior cortex. It is characterized by a short primary neurite which bifurcates; the two branches extend into the circumoesophageal connectives and further to the antennal heart. These neurones resemble in location and morphology strongly the well known DUM-neurones which are considered as an ubiquitous element of insect central nervous systems (review: Weevers 1985). As all DUM-neurones which were biochemically investigated contained octopamine (cf. Evans 1985; Orchard and Lange 1985), it seemed natural to expect this biogenic amine in the antennal heart as well.

We detected octopamine in extracts of antennal hearts using two methods. The one being dansylation with thin layer microchromatography (see Neuhoff 1973). More precise, however, was the radio-enzymatic assay that makes also quantitative results possible. The assay was performed according to the common method of Molinoff et al. 1969. The resulting product was verified with high performance liquid chromatography.

Fig. 3a gives the octopamine values separated according to sex. There is a drastic difference in the octopamine content between the sexes, females contained only a third as much as males. Furthermore we tried to determine the distribution of octopamine within the antennal heart. The question was: is it located in only those axons innervating the muscles or is it mainly in the neurohaemal areas in the wall of the ampullae? For this reason we cut one ampulla close to its connection to the muscle and determined the octopamine content of it and the remaining dilator muscle plus ampulla (Fig. 3b). It was found that more than 40% of the total octopamine content are in the isolated ampulla, that means in the neurohaemal area.

In order to give an idea of the relative octopamine concentration in the antennal heart, we compiled a list of values expressed in pg/mg wet weight from various insect tissues from literature (Fig. 4). Comparing these concentrations it became apparent that no value comes even close to that of the male antennal heart in the cockroach. One must not forget that this value refers to the entire organ, so that the concentration in the ampullae alone must be much greater.

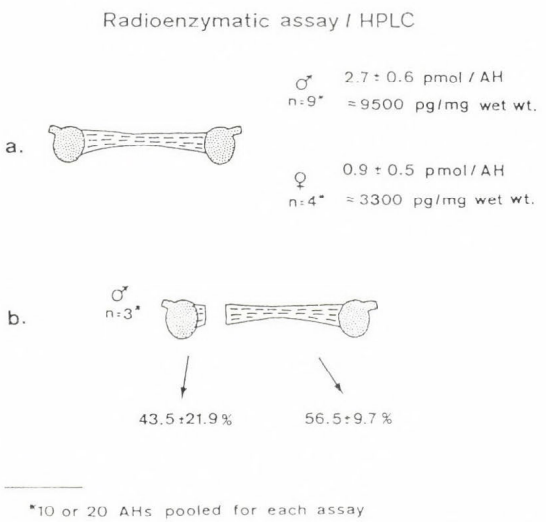


Fig. 3. Results of octopamine determination in the antennal heart of *Periplaneta americana* by the radioenzymatic assay. **a** whole organ of both sexes **b.** percentage for isolated ampulla and the other ampulla plus dilator muscle.

Nervous tissue[a]	pg/mg wet weight
Cerebral ganglion	1500 - 4500
Thoracic gangilon	500 - 1500
Corpora cardiaca[b]	4500
Muscle[c]	
Non-octopaminergic innervated	20 - 100
Octopaminergic innervated	2000
Haemolymph[d]	pg / µl
Resting level	5 ≈ 30 nM
Mechanical stress, 1 min	15 ≈ 90 nM

Fig. 4. Concentration of octopamine found in various insect tissues.
Quoted from: *a* Evans 1985, Davenport and Wright 1986 (various species, including *Periplaneta*). *b* calculated from Evans' 1978 value in pmol/organ on the basis of the weight of 0.04 mg/pair corpora cardiaca (*Periplaneta*). *c* Orchard and Lange 1985 (*Locusta*). *d* Davenport and Evans 1984; Bailey, Martin and Downer 1983 (*Periplaneta*). Some values rounded.

What role does octopamine play in the antennal heart? The extraordinarily high concentration strongly suggests that octopamine is directly released from the terminals into haemolymph, and does not, as one could alternatively suspect, function there as a local mediator for the release of another neurohormone. A release of high amounts of octopamine into haemolymph could be demonstrated under various stressful circumstances. The elevation takes place very quickly and there are good indications for a neurohormonal role of octopamine in insects beside that of a modulator or transmitter (see Orchard 1982, Evans 1985, Hoyle 1985).

The antennal heart's function as a neurohaemal organ deserves special interest from a functional point of view. Substances released there are pumped into the antennae. These appendages are extremely long in cockroaches. In male adults of *Periplaneta americana* they are 6.5 cm long, which is more than their own body length. The circulation period in these appendages is considerable and lies between 10-15 minutes, as can be estimated from observations of haemocyte movement. Substances released from the neurohaemal areas into the ampulla lumen are immediately pumped into the antennae and it is obvious, when one considers this long circulation period, that they must have their target sites within the antennae itself.

Looking at the anatomy of the antenna more closely, one can narrow down the number of possible target sites for the neurohormones of the ampulla. In all pterygote insects the antennal flagella contain only little tissue. Most conspicuous are the sensory neurones, 90% of which are chemoreceptors in *Periplaneta* (Schaller, 1978). Additionally, there are only a few tracheae and epidermal cells. With regard to this anatomy the sensory apparatus immediately suggests itself as a suitable target for hormonal regulation. In particular, a modulation of receptor sensitivity is a conceivable and intriguing possibility. Evidence is accumulating, namely, that contrary to general opinion, a modulation of sensitivity exists in insect chemoreceptors, similar to that well known for some other sensory systems (Blaney et al. 1986).

Another target area for a hormonal regulation by the substances of the antennal heart could be the transport epithelium in the proximal portion of the antennal vessel. As these cells are probably involved in the regulation of the chemical composition of antennal haemolymph, this again could well be of particular importance for the antennal sensory apparatus. The schematic diagram (Fig. 5) summarizes the possible functional relationships for the antennal heart as a

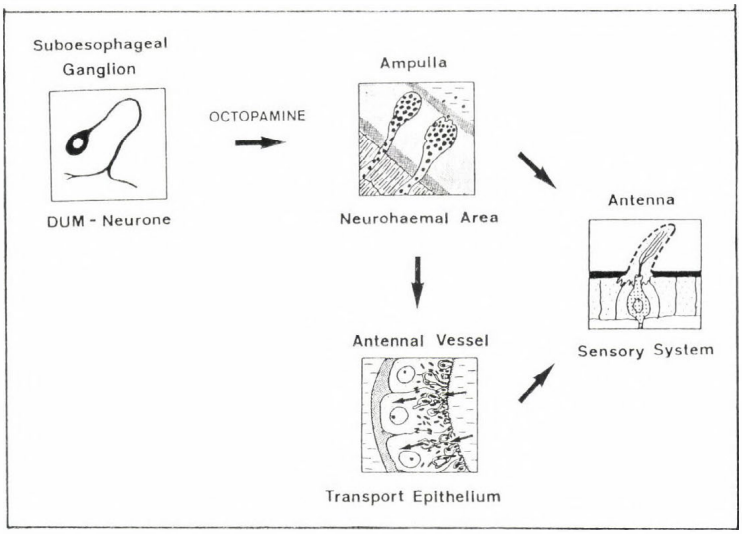


Fig. 5. Possible relationships for the function of the antennal heart of *Periplaneta americana* as a neurohaemal organ.

neurohaemal organ. Regardless of whether the sensory cells or the transport epithelium are the primary target site, the presented results allow useful working hypotheses for further sensory physiological research.

Acknowledgements: Supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (P 5790, P 6088M) and grants of the German Democratic Republic.

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DISCUSSION

HONEGGER, H.W.: During antennal movements long antennae as the one of cockroaches have to be stiffened. Do you think octopamine also could act on the antennal hearts to increase their contractions?

PASS, G.: In cockroaches the antennal heart is not essential for stiffness of the antennae, as one can see after damaging these circulatory organs. However, in the cockchafer beetle Melolontha I found that the antennal heart is involved in the well-known spreading of their lamellate antennae by elevating the haemolymph pressure in the antennal haemocoel. To your second question: We have indications that octopamine is indeed involved in the control of the antennal heart rhythm, but in an inhibitory way. In this case, however, the octopamine must be released not from the neurohaemal areas in the ampullae but by axon terminals on the dilator muscle. This topic is considered in greater detail in another lecture of this symposium (Hertel, Pass and Penzlin).

KUTSCH, W.: Is there any correlation between the octopamine concentration in any of your systems and age or developmental stage?

PASS, G.: This is not tested so far.

SALÁNKI, J.: Do you have any explanation to the fact that octopamine concentration in the antennal heart of males was about 3 times higher than in females?

PASS, G.: The only difference between the antennae of both sexes is their sensory equipment. Males possess about twice as much receptor cells as females and furthermore sensilla types which are lacking in females. The reason for the differences in the octopamine content between sexes should therefore probably be searched in this context.

Symposia Biologica Hungarica 36 (1988)

THE EFFECTS OF THE NEUROPEPTIDE PROCTOLIN AND OF OCTOPAMINE ON THE ANTENNAL HEART OF PERIPLANETA AMERICANA

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ABSTRACT. The antennal heart of the cockroach *Periplaneta americana* consists of two ampullae joined by a dilator muscle contracting in vitro with 21.6 ± 8.2 beats min^{-1}. This rhythm shows a myogenic automatism. Stimulation of the antennal heart nerve results in a transitory block followed by an acceleration of the beat rate. The neuropeptide proctolin has a strong excitatory effect on the heart rate by increasing the steepness of the pacemaker activity. Moreover it produces a slight depolarisation. The ED_{50} for proctolin is about 10^{-7} M. In contrast, octopamine cancels the antennal heart rhythm with an ED_{50} of about 10^{-6} M. The inhibition is caused by a strong hyperpolarisation, indicating an action mechanism via an increase of potassium conductance. This assumption is also supported by pharmacological experiments. The possible roles of proctolin and octopamine in vivo are discussed.

INTRODUCTION

Insects possess, in addition to the large tubular heart ("dorsal vessel"), small accessory pumps to effect haemolymph circulation in long body appendages. The physiology of these organs has been poorly studied so far, although they deserve special interest as relatively simply organized nerve-muscle systems (review: Miller 1985). The best investigated accessory circulatory pump is the antennal heart of the cockroach *Periplaneta americana*. This organ consists of two ampullae located in the head, from which vessels leading into the antennae arise. The ampullae are interconnected by a rhythmically contracting transverse dilator muscle (Fig. 1; Pawlowa 1895; Pass 1985). Intracellular electrophysiological recordings of this muscle (Hertel et al. 1985) showed pacemaker activities. This suggests a myogenic

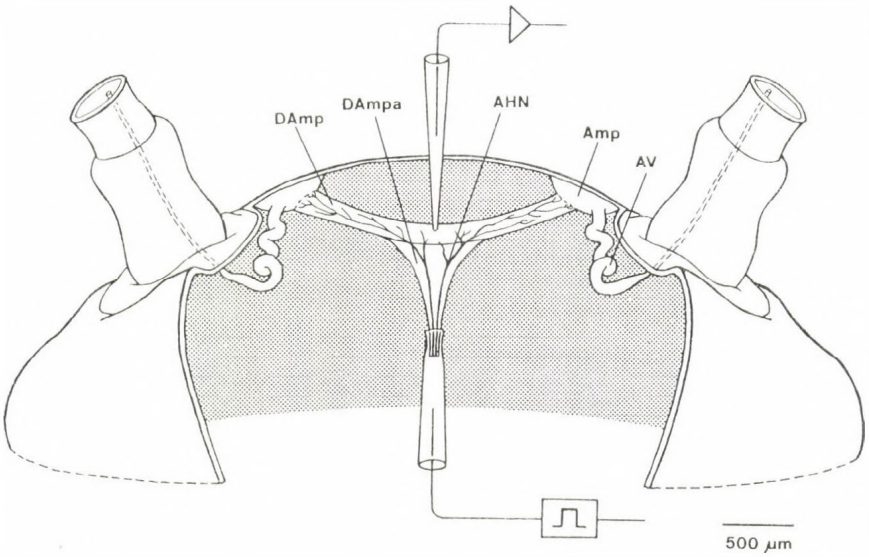

Fig. 1. Morphology of the antennal heart of *Periplaneta americana* showing the sites for intracellular recording of cardiac muscle fibres and for electrical stimulation of the antennal heart nerve by a suction electrode. *Amp* ampulla, *AHN* antennal heart nerve, *AV* antennal vessel, *DAmp* dilator muscle of ampullae, *DAmpa* accessory dilator muscle of ampullae.

automatism for the antennal heart similar to that found for all dorsal vessels of insects examined so far (review: Miller 1985). The antennal heart dilator muscle is innervated (Pass et al. 1987a) indicating additional neuronal control.

The aim of the present study was to elucidate some of the neuronal and/or hormonal regulatory mechanisms involved in this myogenic rhythm. It includes, on the one hand, stimulation experiments of the antennal heart nerve and, on the other hand, various pharmacological studies with putative transmitter and/or modulators.

MATERIAL AND METHODS

For the experiments the antennal hearts of adult male *Periplaneta americana* L. were isolated from the head together with a part of the cuticle and preserved in vitro

in a HEPES-buffered saline (cf. Hertel et al. 1985). Heart beat frequency was recorded with either a mechanical or an impedance transducer apparatus. Stimulation of the antennal heart nerve was done via the cut nerve by means of a suction electrode, and for intracellular recording of heart muscle fibers flexible microelectrodes were used (Fig. 1).

RESULTS AND DISCUSSION

Electrical stimulation (30 Hz, 2 ms, 5-10 V) of the antennal heart nerve showed a biphasic effect in the contraction rhythm of the dilator muscle in more than 80% of the preparations: the heart beat was immediately slowed or blocked for a short time, followed by a temporary acceleration. Both effects depended on stimulus intensity (Fig. 2).

Intracellular recordings revealed that the inhibitory effect after short stimulation (4 pulses) was accompanied by a stop of pacemaker activity with a slight tendency to hyperpolarization (Fig. 2d). The block after long lasting stimulation (5s and 10V) was accompanied by a stop of pacemaker activity as well, but with a much more intense turning back of the potential. In 60% of the cases the potential passes over the point of maximal repolarization up to 11.1 mV (Fig. 2e). This inhibitory effect continued in Cl^--free solution (Fig. 2f). The potassium channel blocker 4-aminopyridine ($5x10^{-4}$ M to 10^{-3} M) suppressed the block normally evoked by electrical stimulation (Fig. 3). Phentolamine ($5x10^{-6}$ M) known as an α-adrenergic antagonist had a comparable effect.

Among the transmitter candidates tested, octopamine was the only one with a distinct inhibitory effect on the antennal heart beat. The well-known inhibitory transmitters GABA and glycine were completely ineffective. The typical effect of octopamine on the antennal heart consisted in a transitory block, followed by a reduced beat rate (Fig. 4). Despite the considerable variability of the octopamine effects a dose-response relationship for both the duration of the heart block and the subsequent reduction of heart rate could be evaluated (Fig. 5). The threshold concentration was about 10^{-8} M; in about half the cases, concentrations higher than 10^{-5} M resulted in a a block for the duration of the test (6 min). The ED_{50} for octopamine was about 10^{-6} M.

In order to characterize the octopamine receptors the agonists clonidine, naphazoline and tolazoline and the antagonists phentolamine, gramine,

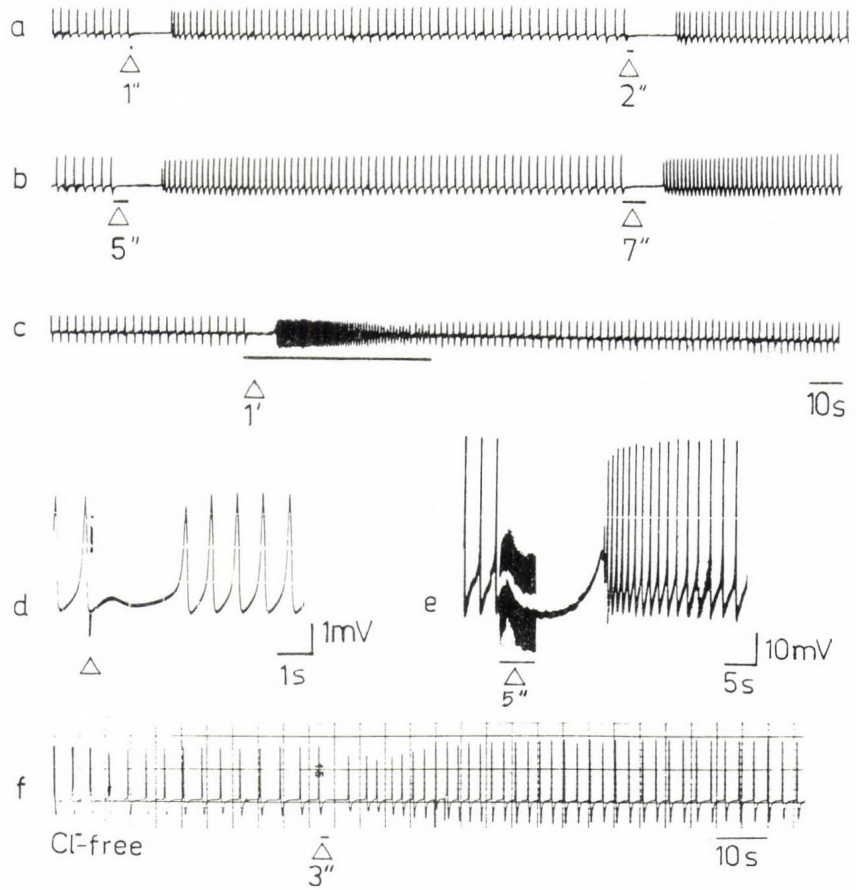

Fig. 2. Electrical stimulation of the antennal heart nerve results in a transitory beat block followed by a temporary acceleration. **a-c.** The extent of the biphasic effect depends on stimulus duration (30Hz, 2 ms and 10V; mechanocardiograms). **d.** a short stimulus (4 pulses) results in an IPSP. **e.** a 5 s stimulus is followed by hyperpolarisation (d and e are intracellular recordings). **f.** The effect is also present in Cl^--free saline (mechanocardiogram).

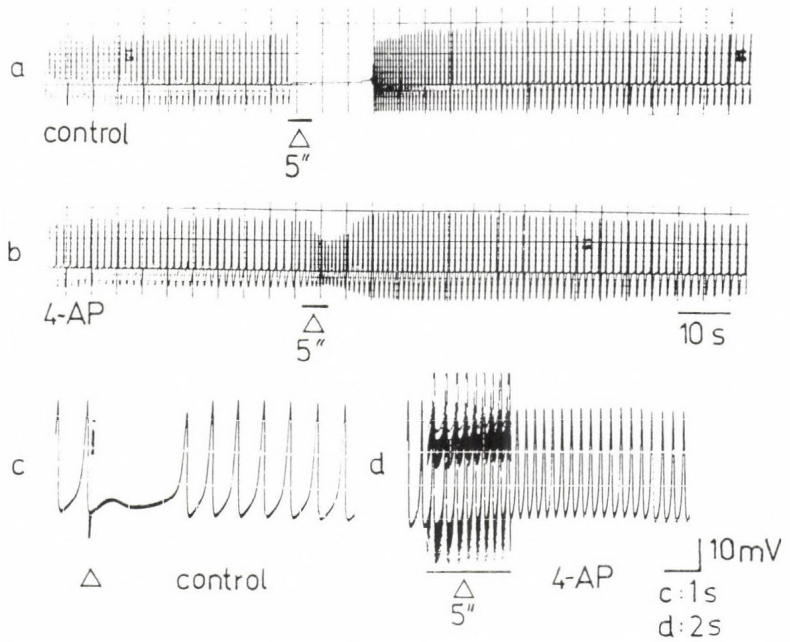

Fig. 3. The potassium channel blocker 4-aminopyridine (*4-AP*; 10^{-3} M) cancels the inhibitory effect normally seen immediately after electrical stimulation of the antennal heart nerve (a and b mechanocardiograms, c and d intracellular recordings).

metoclopramide and chlorpromazine were tested on the antennal heart. None of the antagonists tested (clonidine $5x10^{-5}$ M to $2x10^{-4}$ M, tolazoline $5x10^{-5}$ M to $5x10^{-3}$ M, and naphazoline $5x10^{-5}$ M to 10^{-3} M) had a significant effect on the beat rate. Among the antagonists chlorpromazine ($5x10^{-5}$ M) was most effective, completely cancelling the effects normally evoked by octopamine ($5x10^{-6}$ M) (Fig. 4a). Metoclopramide and gramine in the same concentration gave similar results (Fig. 4b,c). In the presence of these two antagonists, octopamine ($5x10^{-6}$ M) caused only a slight reduction of heart beat rate, but no block. Phentolamine ($2.5x10^{-5}$ M) was an active antagonist, too, but was less effective.

Evans (1981) distinguished two main types of octopamine receptors in the extensor muscle of the hindleg tibia of *Schistocerca*. He reported that at the octopamine-1 receptor clonidine was a more effective agonist than naphazoline, and chlorpromazine a better antagonist than metoclopramide. At the octopamine-2

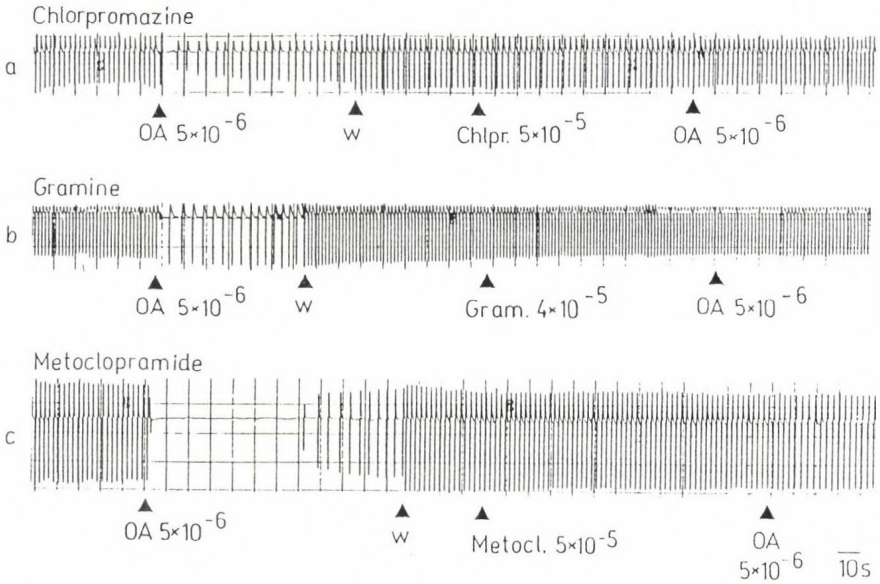

Fig. 4. The effects of some octopamine antagonists on the antennal heart beat (mechanocardiograms). The antagonists were tested after a control test with octopamine (*OA*) which was washed out with saline (*W*). **a.** Chlorpromazine cancels completely the inhibitory octopamine effect. **b and c.** Gramine and metoclopramide are very effective antagonists too, but a slight inhibitory effect on the heart beat rate is still present after addition of octopamine.

receptors the situation in respect to the effectiveness of these substances was exactly vice versa. Furthermore, the octopamine-2 receptors are selectively blocked by gramine (Evans 1981; Pannabecker and Orchard 1986). In the antennal heart, however, none of the tested agonists were effective. Chlorpromazine, the most effective octopamine-1 receptor antagonist, was also the most effective antagonist in the antennal heart. However, gramine, which is considered to be a specific octopamine-2 receptor antagonist, was effective too. Therefore we must assume the existence of an additional octopamine receptor type in insects different from those described by Evans (1981) (see also Orchard et al. 1982; Orchard and Lange 1986).

Intracellular recordings showed that the block evoked by octopamine $5x10^{-4}$ M was accompanied by a strong hyperpolarization of 13.6 + 1.2 mV (n = 14) (Fig. 6a). Similar to the situation seen in the nerve stimulation experiments, the inhibitory

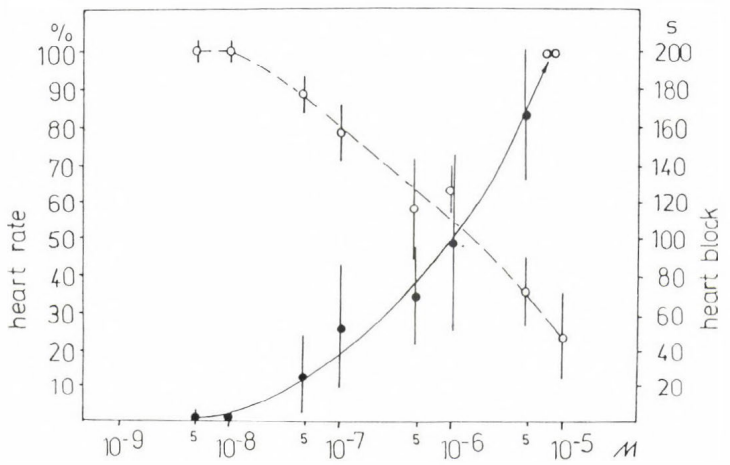

Fig. 5. Dose-response curves for the octopamine effects on the antennal heart. An elevation of the concentration results in a decreasing heart beat rate (circles and broken line) but the duration of the beat block is increasing (solid circles and solid line).

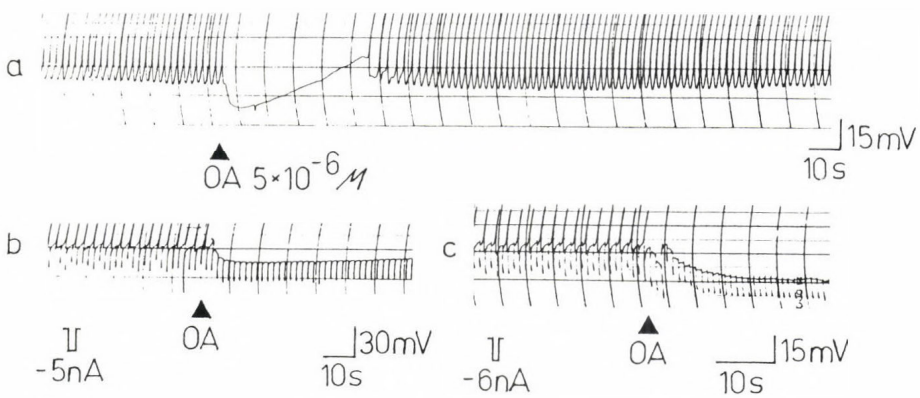

Fig. 6. a. Intracellular recordings of muscle fibre potentials reveal a strong hyperpolarization after the addition of octopamine (*OA*; $5x10^{-6}$ M). **b and c.** The input resistance is reduced after addition of octopamine ($5x10^{-5}$ M), which is demonstrated by the shortening of the downward deflections due to the passing of hyperpolarizing current pulses.

effect of octopamine was still present in Cl^--free saline, but absent in the presence of $5x10^{-4}$ M 4-aminopyridine. This indicates that an increase in potassium conductance may be involved in the action of octopamine. Preliminary results further indicate that octopamine reduces the input resistance up to 40% (Fig. 6b,c) supporting this assumption.

Considering the situation in vivo, it is noteworthy that DUM-neurones were found associated with the antennal heart (Pass et al. 1987a) and that octopamine could be demonstrated biochemically in an extraordinarily high concentration in extracts of the antennal heart (Pass et al. 1987b). The bulk of this substance, however, was detected in the ampullae walls, where numerous neurosecretory fibres terminate. Octopamine and/or other substances released from these neurohaemal areas into haemolymph are immediately pumped into antennae where their target sites may be expected. The similar effects on antennal heart beat following octopamine treatment or immediately after nerve stimulation, however, may indicate an involvement of this biogenic amine in heart beat control too.

The neuropeptide proctolin was found to be a very effective stimulating agent at the antennal heart (Hertel et al. 1985). It enhanced the beat frequency dose-dependently up to 400% at 10^{-6} M (Fig. 7). The threshold concentration was $5x10^{-9}$ M and the ED_{50} about 10^{-7} M. Intracellular recordings showed that the stimulation of the antennal heart rate by proctolin was due to a steeper rise of the pacemaker activity. The depolarizing effect was small (10^{-7} M proctolin: 2.5 $\pm$ 0.7 mV, n = 13; Fig. 8). Both the threshold potential and the form of the action potential remained unchanged. In the presence of TTX (10^{-4} M) the heart rhythm as well as the proctolin response continued. This indicates that TTX-sensitive fast sodium channels are not involved in either the generation of heart muscle potentials or the proctolin action. 4-Aminopyridine mimics the proctolin effect suggesting a mechanism which involves the reduction of the potassium conductance, similar to the situation found in the hyperneural muscle of *Periplaneta* by Hertel and Penzlin (1985, 1986, 1987) and in a molluscan neurone by Richter and Penzlin (1987a,b). However, a diversity in the action mechanism of proctolin in different organs must be taken into account (O'Shea and Adams 1986).

We did not succeed to demonstrate proctolin immunocytochemically in the antennal heart. A possible neurohormonal role of this neuropeptide is conceivable only in a spatially and temporarily limited fashion because of its relatively low concentration and its rapid inactivation in haemolymph (cf. O'Shea and Adams 1986). With regard to the antennal heart it has furthermore to be taken into account

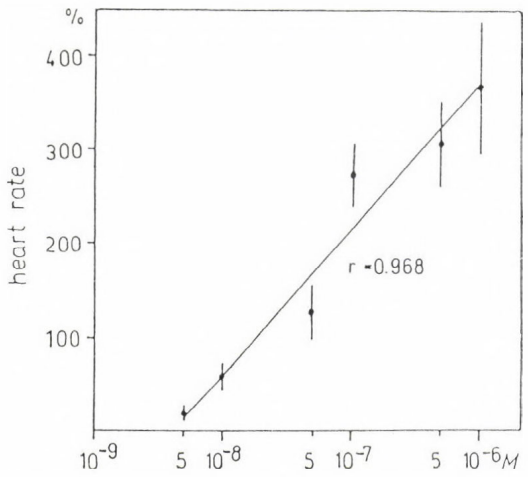

Fig. 7. Dose-response curve for the proctolin effect on the antennal heart rate.

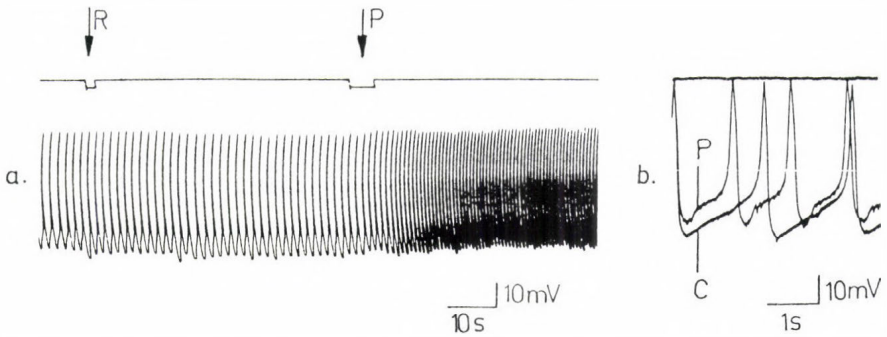

Fig. 8. a. Proctolin (*P*) stimulates the heart beat rate and produces a small depolarization (intracellular recording). **b.** The same recording as in 8a on faster time base. Control recording (*C*) and recording after proctolin addition (*P*) are superimposed and show the steeper rise of the pacemaker activity caused by proctolin.

that proctolin could not yet be demonstrated in higher concentrations in the cockroach head (Bishop and O'Shea 1982). Nevertheless, preliminary experiments about the influence of pronase treated and untreated antennal heart homogenates on the frequency of intact antennal hearts indicate the existence of a stimulatory peptid factor in beat control which may be biochemically similar but not identical with proctolin.

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DISCUSSION

HONEGGER, H.W.: Do you know the source of proctolin which acts on the antennal heart muscles? Do you know any proctolinergic neurons projecting to the antennal heart?

PENZLIN, H.: We did not succeed in demonstrating immunocytochemically proctolin-like activity in our system. Nevertheless, preliminary experiments indicate that the antennal heart is under the control of a peptide which may be biochemically similar to but not identical with proctolin.

KISS, T.: It was interesting to hear about transmitter function of proctolin in Periplaneta heart. We also studied the effect of proctolin in Locusta heart, namely on postsynaptic potentials. From these studies we supposed that proctolin is a modulator or acts on contractile apparatus. What is your opinion in this respect?

PENZLIN, H.: You are right in so far as in contrast to the "classic" transmitters proctolin does not affect the postsynaptic cell primarily by a drastic change of the membrane potential. So there is a clear difference. It is now a question of definition whether we use in this case still the term "transmitter" or not.

KUTSCH, W.: Since Dr. Pass indicated a possible modulation of the antennal receptor system by octopamine there seems to arise a contradiction: with octopamine increase the heart muscle activity is depressed. Therefore the increased octopamine concentration can no longer circulate through the antenna.

MODULATION, INTEGRATION AND LEARNING

Symposia Biologica Hungarica 36 (1988)

PRE- AND POSTSYNAPTIC ACTIONS OF FMRF-NH_2-LIKE PEPTIDES IN INSECT NEUROMUSCULAR SYNAPSE+

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SUMMARY

FMRFamide and YGGFMRFamide potentiate neuromuscular transmission in locust jumping muscle. Apart from directly affecting excitation-contraction coupling these peptides as well as a factor extracted from a neurohaemal organ lead to an increased amplitude of the excitatory junction potentials evoked by stimulation of the slow excitor axon (SETi). Furthermore the muscle fibre membrane is depolarized and its resistance is increased. (YGG)FMRFamide was practically ineffective in various non-insect neuromuscular preparations. Using a 'macro patch clamp'- technique direct evidence is obtained for a peptide induced increase in the evoked transmitter release. This is found even if Na^+-channels are blocked. The depolarization and increase in membrane resistance of the muscle fibre cannot be accounted for by an effect on the Cl^--conductance but most probably result from reduction of a K^+-conductance.

INTRODUCTION

The molluscan cardioexcitatory peptide FMRFamide (Price and Greenberg, 1977) modulates neuromuscular transmission at locust skeletal muscle in several ways: it increases the amplitude of excitatory junction potentials (ejps); it depolarizes the muscle fibre; and in addition it renders excitation-contraction coupling more effective (Walther et al., 1984). From a variety of structurally related amidated peptides YGGFMRFamide has so far proven to be the most potent - about 100 times more potent than

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FMRFamide - while peptides having either the C-terminal sequence -Asp-Phe-NH_2, e.g. Cholecystokinin (CCK) - octapeptide, or the sequence -Arg-Tyr-NH_2,e.g. bovine pancreatic polypeptide (BPP) and neuropeptide Y, are practically ineffective (Walther et al., 1984; Evans and Myers, 1986b; for a recent review of these and further results see Evans and Myers, 1986a). These investigations thus indicate the presence of muscular non-synaptic receptors highly specific for -RFamide and in addition suggest that such receptors occur in the presynaptic axon terminals as well. The presence of FMRFamide- and/or BPP-like materials has been demonstrated in a variety of neurons of the locust ventral nerve cord by immunocytochemical studies (Myers and Evans, 1985a). Subsequent investigations gave evidence against BPP-like and in favour of -RFamide -like peptides occurring in these neurons. These apparently are all interneurons or neurosecretory cells but not motor or sensory neurons (Myers and Evans, 1985b; Walther and Schäfer, in press).

The suggestion that one or more -RFamide-type peptides are released from neurohaemal organs and thus can act as neurohormones (Evans and Myers, 1986a) has recently been given strong support by a detailed comparison of the neuromuscular effects of YGGFMRFamide and of extracts from a metathoracic neurohaemal organ (Walther and Schiebe, 1987a): Both agents produce the same pattern of effects on the ejps, on the non-synaptic muscle fibre membrane and on the amplitude and time course of the contractions. They further both act rather slowly - the effects are maximal only after a few minutes and are reversed only after prolonged washing - which points to the involvement of second messenger(s). In the present paper we describe results from electrophysiological studies concerning the mechanism of action of YGGFMRFamide and related peptides both on the motor nerve terminals and on the muscle fibre membrane. Some of the results have been previously published in abstract form (Walther et al., 1985; Walther and Schiebe, 1987 a,c).

MATERIALS AND METHODS

Experiments were carried out at room temperature on distal fibre bundles of the M. extensor tibiae (ventral side) from adult locusts (Schistocerca gregaria). For the investigations on transmitter release usually nerve 5 of the metathoracic ganglion (cf. Hoyle, 1955) was severed 7 to 10 days prior experiment in order to eliminate synaptic transmission from the ter-

minals of the fast excitatory axon (FETi) without impairing transmission from the slow excitor axon (SETi). In other animals 24-48 h before use the hindlegs received an injection of a wasp venom (species Habrobracon hebetor) which blocks excitatory but not inhibitory neuromuscular transmission (Walther and Rathmayer, 1974). Details of the preparation, solutions and conventional procedures of stimulating and recording were as previously described (Walther et al., 1984; Walther and Schiebe, 1987b).

For focal extracellular stimulation and recording the method introduced by Dudel (1983) was used which involves a modified "patch clamp" circuit (supplied by Zeitz-Electronics, Augsburg, FRG). Macropipettes were used which had inner tip diameters of 30-50 µm (mostly 35 µm). In order to locate nerve terminals of the SETi the axon was stimulated and the pipette moved along the surface of a muscle fibre. Once a weak negative going deflection indicated the presence of a synaptic region - frequently near a fine tracheal branch - the pipette was repositioned until the response was maximal. Subsequently it was pressed mildly and usually without suction against the muscle fibre. Stimulation was performed through the pipette by negative going rectangular current pulses of 1 ms duration and varying amplitude. Then the pipette was fine-positioned. Once located, a synaptic site was considered suitable for an experiment if (1) spontaneous miniature excitatory junctional currents (mejcs) were well above the noise level (Fig.1) and occurred at a frequency far below 1/s; (2) a current pulse amplitude of 0.5-1 µA was sufficient to evoke release at about half of the number of stimuli. The stimulation was run continuously at a rate of 0.25-1/s.

Under these conditions release is probably stimulated only from a fraction of the terminal very close to the center of the pipette. This is concluded from an experiment with combined extra- and intracellular recording of the synaptic responses evoked by stimulation via a 35µm patch pipette: If the amplitudes measured intracellularly (ejps) are plotted against those measured extracellularly (ejcs), most data points lie within a narrow zone, i.e. they are well correlated, but some 5-10% deviate in a manner indicating that too small an extracellular response had been measured. If the spontaneously occurring miniature synaptic signals from the same experiment are plotted the same way about 60% show this type of deviation. This can be explained by the assumption

that spontaneous release occurs anywhere from the stretch of nerve ending covered by the patch pipette but that there is a decrement in recorded amplitude from the center to the rim.

If there are some 25 synaptic sites located on a 30 µm nerve ending (which seems reasonable from electron- and light-microscopical experience) the order of 10 stimulated sites in the center of the pipette-covered area can be derived from the above figures for spontaneous signals. This is compatible with the rough estimate of the number of release sites (n) derived from the release statistics (cf.Results).

Drift was a serious difficulty with the extracellular approach, at least in this preparation where nerve terminals mostly extend within the clefts between muscle fibres rather than on their free surfaces. The mean quantal content of the evoked ejcs tended to increase or decrease over the time periods (>30 min) required to conduct an experiment. The reasons for this drift are not clear. They may reside in minute changes of the position of the electrode relative to the nerve terminal without a clear change in "seal" resistance as tested by a short command pulse to the clamp amplifier. Because of this problem the following precaution was met: About 10 min and 5 min prior to the envisaged application of peptide the average response to 50 stimuli was determined. The application was performed only if the average from the second measurement did not deviate by more than 10% from that obtained from the first measurement and only if the drift was towards a reduction but not an increase of the response. From the experiments with over 20 preparations only 6 were successful.

RESULTS AND DISCUSSION

Ineffectiveness of (YGG)FMRFamide in various non-insect neuromuscular preparations

Both from the comparative physiological viewpoint and because of certain limitations which complicate neuromuscular studies in insects it was of interest to find out whether (YGG)FMRF-amide is active in other preparations as well. For this purpose contractions or endplate potentials (epps) reduced to a few millivolts by curare or magnesium in frog and mouse muscles (M.cutaneus pectoris and M.soleus, respectively) were investigated (together with H.Lorković). No consistent effects were

obtained by application of either peptide up to a concentration of 5×10^{-6} M. In accord with this the presynaptic axonal currents in the mouse M.triangularis sterni-preparation (e.g. Penner and Dreyer, 1986) were not affected by FMRFamide (Penner and Dreyer, personal communication). Somewhat surprisingly, both FMRFamide and YGGFMRFamide had little or no effect on muscle contraction and ejps in various crustacean preparations: claw opener of Procambarus (tested up to 10^{-7}M) and Cancer (tested up to 10^{-5}M); gastric muscle of Callinectes (tested up to 10^{-5}M). In the lobster a FMRFamide-related octapeptide from the nervous system acts quite potently on transmission in an abdominal wall muscle (Kobierski et al., 1987) whereas FMRFamide has only a very weak effect (E.Kravitz, personal communication).

Effects of YGGFMRFamide on transmitter release

Previously, only indirect evidence had been obtained that YGGFMRFamide enhances transmitter release in locust jumping muscle: Although the ejp amplitude is enhanced in the presence of peptide the sensitivity of the transmitter receptors is not altered since 1) the synaptic response to bath applied pulses of glutamate was not affected by YGGFMRFamide (Walther et al., 1984) and 2) the amplitudes of the mejps were not increased (Evans and Myers, 1986b). Furthermore the coefficient of variation of the potentiated ejps is smaller than in the absence of peptide which points to an increased rate of release (Walther and Schiebe, 1987b). There is also an increase in mejp-frequency yet this does not prove that the neurally evoked release is enhanced as well. Even the opposite can be true as in the case of certain drugs or venoms (e.g. Walther, 1980).

In order to gain quantitative information on the presynaptic modulation focal extracellular stimulation and recording of ejcs (cf. Methods) was carried out. This has the advantage that quantal analysis of the responses can be carried out even under such ionic conditions where conduction of nerve impulses is blocked. In the partially denervated preparations (cf.Methods) used for the extracellular technique, interacellular recording indicated that the peptide induced potentiation of the ejps was similar to that known from untreated animals. Fig. 1 shows examples of focally recorded spontaneously occurring and evoked ejcs. In the presence of YGGFMRFamide the number of failures (i.e. sti-

muli not leading to release of transmitter; e.g. Fig.1a), is reduced as expected for a presynaptic effect (cf.Fig.2). Some of the terminals did not respond to the application of peptide. Perhaps in such cases the peptide did not reach the terminal under the patch pipette. We cannot exclude, however, that some terminals are in fact unresponsive.

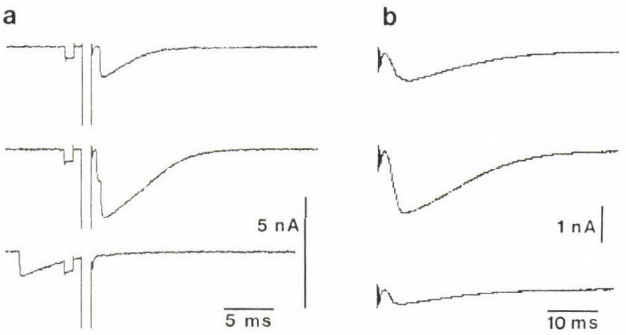

Fig.1. Excitatory junctional currents.
a, single records; release of 1,2 and 0 quanta in upper, middle and lower trace, respectively; the latter also shows spontaneous release of a quantum preceding a small test pulse and the stimulating pulse (out of range). b, averaged responses to 50 stimuli, applied before, during and after application of 2.5×10^{-7}M YGGFMRFamide into the bath. The release gradually declined during the experiment but was increased by the peptide (cf. text and Fig.2). $[K^+]_o$ = 10mM.

Amplitude histograms (like those of Fig.2) indicate that the size of the evoked unitary ejcs is not affected by the peptide - in accord with the above concluded lack of a postsynaptic effect. Thus it is possible to derive the increase in mean quantal content (m), caused by peptide, by relating the average amplitude (failures included) measured in the presence (A_2) and absence (A_1) of peptide (i.e. $\Delta m = (A_2/A_1 - 1) \times 100$). This way Δm was found to be 105 % $\pm$ 15 (SEM; 4 experiments) for a concentration of 10^{-7}M.

In two experiments the amplitude histograms had fairly clear peaks (like in Fig.2) so that it was possible to determine how often 1,2 or more quanta had been released. m calculated this way was 0.5 to 0.6 in the absence of peptide and Δm was 108 $\pm$ 7%, which is close to the figure of

103 ± 17% derived from the averaged responses of the same experiments. The method of failures which assumes Poisson statistics (Del Castillo and Katz, 1954) and which does not require a perfectly stable amplitude of the unitary ejc, yields very similar m-values in the absence of the peptide but ~10% higher values in its presence. According to theory (Johnson and Wernig; 1971) with a higher rate of release such deviation can be expected. From m and its variance it can be deduced whether an increase in release is due to a change in the average release probability (p) or the number of releasing sites (n) or both (Ginsborg, 1970). The results from analyses of the data shown in Figs 1, 2 and from a second experiment are compatible with the notion that the peptide increases p (which is of the order of 0.1 before application of peptide) whereas a conclusion with regard to possible changes of n (without peptide supposedly around 5) cannot be drawn at present.

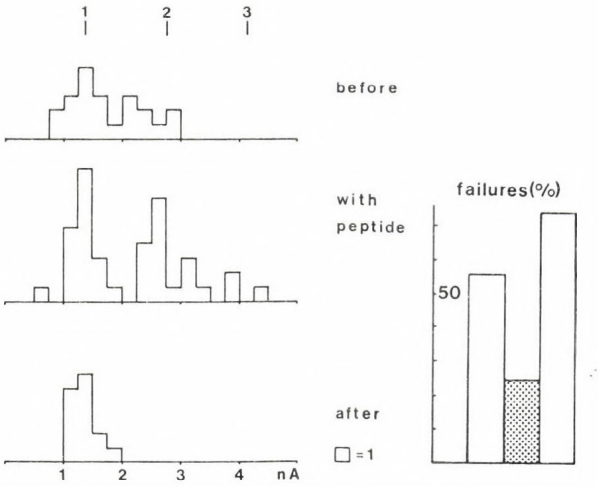

Fig.2. Amplitudes of excitatory junctional currents in the presence and absence of YGGFMRFamide.

From the same experiment as in Fig.1b. Numbers above upper histogram indicate the peak positions corresponding to releases of 1, 2 or 3 quanta. The histogram to the right indicates the frequency of failures which was reduced in the presence (stippled bar) of peptide. From the failures and the number of stimuli, i.e.50 in each case, m-values of 0.6, 1.4 and 0.3 are derived according to Poisson statistics. After washing off the peptide the rate of release was reduced beyond the initial level but the amplitudes of unitary ejcs remained unchanged.

The release of transmitter was also evoked if nervous conduction was blocked by 10^{-6}M tetrodotoxin (TTX). TTX itself reduced the release by about 50% (n = 3) which indicates that our conditions of stimulation lead to some activation of sodium channels in the nerve terminal. The potentiation of release by $2.5x10^{-7}$M YGGFMRFamide, however, was practically the same, i.e. 97 $\pm$ 6% (n = 2), as in the absence of TTX (101 $\pm$ 4; same experiments). Therefore it is unlikely that the peptide produces its presynaptic effects by an action on the Na^+-channels.

Which ionic conductance in muscle membrane is affected by (YGG)FMRFamide?

Application of FMRFamide-like peptides leads to a depolarization which is closely paralleled by an increase in input resistance (R_{in}) (Fig.3). This increase can also be seen from the decay phases of ejps with certain precautions and this way it was found that a 10% increase in membrane resistance (R_m) correlates with ~1 mV depolarization (Walther and Schiebe, 1987b). As in the case of the peptide-effects on ejps and contractions YGGFMRFamide acts (at least 10-times) more potently than FMRFamide. The

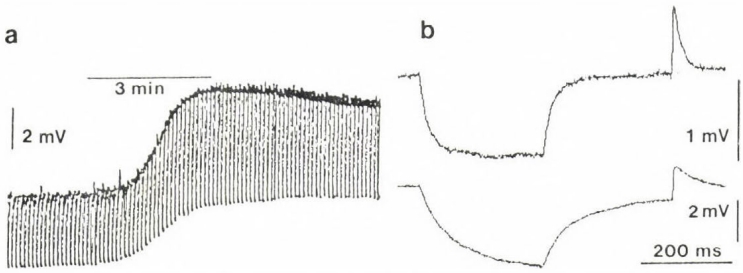

Fig.3. Conductance decrease caused by YGGFMRFamide in muscle membrane. a, continuous recording of hyperpolarizing responses to injection of 5nA-current pulses of 2.5 s duration. For the 3min- period indicated by the horizontal bar $5x10^{-7}$M YGGFMRFamide was applied which caused ~5mV depolarization and ~60% increase in input resistance. b, averages of 12 records showing hyperpolarizing response to a 0.5nA- current pulse and a subsequently evoked depolarizing inhibitory junction potential in the absence (upper trace) and presence (lower trace) of 10^{-8}M YGGFMRFamide. The application of peptide caused ~150% increase in input resistance and a depolarization of ~14mV. Excitatory transmission was blocked by pretreatment with Habrobracon-venom (cf.Methods). a and b: Membrane potential prior peptide application -61 and -62 mV; K^+-concentration 5mM; p_H = 7.2; KCl-electrodes; temperature 21.3 and 20.4°C, respectively.

non-amidated analogues of these peptides are inactive. With YGGFMRFamide the threshold is well below 10^{-8}M. Saturation was not tested since concentrations higher than 10^{-7}M often lead to a depolarization beyond the threshold of contraction. The depolarizing action does not seem to desensitize within time periods of up to 10 minutes. In fibres solely innervated by FETi we did not observe peptide-induced depolarizations.

The depolarizations could result from an inactivation of either chloride or potassium channels which are active at the resting membrane potential. However, an involvement of Cl^--channels is quite unlikely, since in a "Cl-free"saline (i.e. $[Cl^-]_o$ reduced to 10 mM by substitution with methanosulfonate) depolarizations and increases in R_{in} were of the same order of magnitude as in normal saline. This conclusion is also supported by the following experiment: In a (most distally located) muscle fibre which receives inhibitory in addition to excitatory innervation Cl^-channels can be opened by GABA-release from inhibitory nerve endings. If two KCl-electrodes are inserted and hyperpolarizing current pulses are injected through one of them the inhibitory junction potentials (ijps) are consistently depolarizing (Fig. 3b), i.e. E_{Cl^-} is more positive than the resting membrane potential. Under these conditions the peptide-mediated conductance decrease is again accompanied by a depolarization which thus cannot result from closing of Cl^-- channels since this should lead to a hyperpolarization.

The peptides thus seem to inactivate K^+-channels. This is supported by voltage clamp experiments (Walther, in preparation) which demonstrate that the current reduced by YGGFMRFamide reverses at a potential a few millivolts more negative than the resting potential. Effects of FMRFamide-like peptides on K^+-channels have been previously described for gastropod neurons by various authors, e.g. Brezina et al.(1987), Cottrell and Davis (1987), Ruben et al.(1986). In many of these examples K^+-channels were activated but inactivation of some type of K^+-conductance has also been demonstrated (e.g. Cottrell and Davies, 1987). If a decrease in K^+-conductance would also occur in the motor nerve terminal membrane this could explain the potentiation of transmitter release by peptide. It has to be kept in mind, however, that both in muscle and axonal membrane FMRFamide-like peptides could in addition have an effect on Ca^+-currents like in certain gastropod neurons (e.g. Colombaioni et al., 1985). Experiments are in progress to clarify this point.

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DISCUSSION

ALKON, D.L.: Do you attribute the hormone-induced depolarization to reduction of a steady-state K^+ conductance which is activated at the resting membrane potential? Is such a steady-state conductance voltage dependent, particularly at more positive levels of membrane potential?

WALTHER, C.: As to the first point: yes, this is our present working hypothesis. The conductance is not voltage dependent in the range of -55 mV to -100 mV. More positive levels have not yet been investigated.

CARLBERG, M.: Have you tried any K^+-channel blocker yet?

WALTHER, C.: No.

FLOREY, E.: Have you investigated the action of octopamine on e-c coupling in the muscle fibres in which FMRFamide affects this?

WALTHER, C.: In these fibres octopamine, too, increases the rate of relaxation.

MAGAZANIK, L.: 1. Did the peptides change the sensitivity to glutamate application or the amplitude of mejps?

2. How did octopamine affect the input resistance of muscle cells?

WALTHER, C.: 1. Neither the response to bath applied glutamate nor the amplitude of the mejps is significantly changed by the peptides.

2. Octopamine increases the membrane resistance of these muscle cells.

Symposia Biologica Hungarica 36 (1988)

THE ROLE OF TWO PEPTIDERGIC GIANT NEURONS IN MODULATION OF RESPIRATORY BEHAVIOUR IN THE POND SNAIL, LYMNAEA STAGNALIS

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INTRODUCTION

In the freshwater pulmonate snail, *Lymnaea stagnalis*, respiration occurs both by diffusion through the skin and by lung ventilation (Jones, 1961). Lung ventilation is carried out at the surface of the water, and brought about by opening of the pneumostome. Opening of the pneumostome results in gas exchange between the contents of the lung cavity and the atmosphere. When the PO_2 of the water is low, the animal relies for its O_2 supply to a larger extent on lung ventilation. This is achieved by a change in gravity orientation and an increased frequency of lung ventilations. Preventing this type of compensation (for instance by forced submergence) causes a long lasting change in lung ventilation and gravity orientation. When, after a period of hypoxia, the animals are allowed to surface and breathe air, they remain at the water surface for prolonged periods of time, regularly carrying out lung ventilation. Presumably this change is related to the occurrence of an O_2 debt, which develops during hypoxia: the long lasting increase of the duration of lung ventilation is required to meet the increased post anaerobic O_2 demand. Behavioural observations suggest that external O_2 chemosensitivity is involved in mediating the instantaneous responses of the animal upon environmental hypoxia (Janse, 1981). The long lasting changes are probably mediated by internal O_2 chemosensitivity (van der Wilt *et al.*, in prep.).

The respiratory condition of the animal can be viewed as an important motivational state. Apart from respiratory behaviour, a change in O_2 availability has comprehensive effects on other behavioural and physiological processes as well (van der Wilt *et al.*, in prep.). This is reflected in the wide distribution in the central nervous system (CNS) of the O_2 chemosensory input (Janse *et al.*, 1985). Two peptidergic giant neurons, VD1 and RPD2 probably play an important role in the regulation of behavioural and physiological adjustments that occur as a result of changes in O_2 availability. They are extremely sensitive to changes in internal and external PO_2 and branch extensively over the CNS (Boer *et al.*, 1979).

The present paper deals with the neuronal network, involved in the regulation of lung ventilation during environmental hypoxia. Special attention is payed to the role of the two peptidergic giant neurons in the CNS, VD1 and RPD2.

TYPE OF PREPARATION USED

The experiments were performed with semi-intact preparations, consisting of the lung- and mantle area, connected to the CNS by the nerves innervating this area (for a detailed description see Janse *et al.*, 1985). The preparation allows selective exposure of the periphery and the CNS to hypoxia, while simultaneously recording lung ventilatory movements and electrical activity of central neurons. Lung ventilatory movements were recorded using a non-invasive, optical device. Standard electrophysiological equipment was used to record and display intracellular electrical activity of central neurons.

RESULTS

Respiratory movements in the semi-intact preparation

In the semi-intact preparation, decreasing the PO_2 results in pneumostome movements comparable to those observed in the intact animal during environmental hypoxia. Decreasing the PO_2 in the lung-mantle area results in an immediate increase in the frequency of pneumostome opening movements. Decrease of the PO_2 in the saline, surrounding the CNS also results in an increased frequency of opening movements, but the onset of the response is much slower.

Putative respiratory motoneurons

In the visceral ganglion a number of cell types have been identified that cause either closure or opening of the pneumostome upon electrical depolarization. One of these cell types forms a cluster in the rostral part of the dorsal surface of the visceral ganglion. They are provisionally called P1 neurons. Depolarization of one of these neurons causes opening of the pneumostome. Electrical stimulation of the neurons with increasing stimulus intensity results in an increase of the amplitude and the rate of the opening movement and a decrease of the latency (Fig. 1). This suggests a fairly direct effect of these cells on muscles, responsible for opening of the pneumostome.

A decrease of the external PO_2 results in an increase of the spike frequency in P1 neurons. The observed increase of frequency of opening movements of the pneumostome during low PO_2 is probably due to increased firing frequency of these cells.

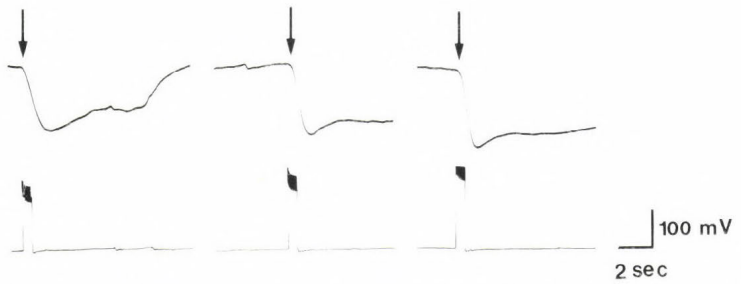

Fig. 1. Intracellular suprathreshold depolarization of a P1 neuron (lower trace) causes opening of the pneumostome (upper trace, arrows). Increasing stimulus intensities result in increase of the amplitude and rate of movement and decrease of the latency of the response.

Interneurons

The firing frequency of P1 neurons is largely determined by two distinct synaptic inputs with opposing effects. The inhibitory input is active during closure of the pneumostome, while the excitatory input occurs during opening (Fig. 2). A large number of neurons in the visceral and right parietal ganglia receive both inputs, which are easily recognizable because of the profound effects on the spike activity of their follower cells. The two inputs invariably

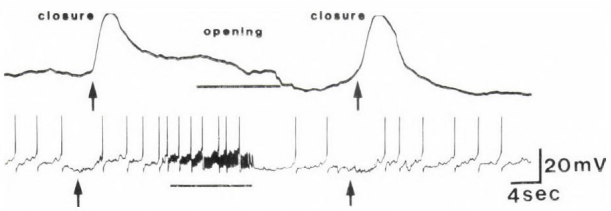

Fig. 2. Prior to closure of the pneumostome (upper trace, arrows) P1 neurons receive inhibitory input (lower trace, arrows), while prior to opening of the pneumostome (upper trace, bar) the neurons receive excitatory input (lower trace, bar).

have opposing effects on their follower cells. Fig. 3 shows the localization in the CNS of neurons, receiving the two inputs. These neurons have been identified by Benjamin and Winlow (1981), and the nomenclature, introduced by these authors is used.

The origins of both inputs are as yet unknown. Presumably they originate from interneurons which exert their effect on the pneumostome indirectly by altering the spiking activity of centrally located motoneurons. This follows from the observation that after blocking synaptic input by exposing the CNS to high Mg^{2+}/low Ca^{2+} saline, pneumostome movements no longer occur, and the two inputs are abolished. The synaptic input that

precedes closure movements of the pneumostome is probably identical to input 3, described by Benjamin and Winlow (1981), and will be referred to as such in the following. The input that precedes opening movement of the pneumostome has not been described previously, and will be referred to as input 5. Neurons that receive input 3 and 5 and cause closure of the pneumostome remain to be identified.

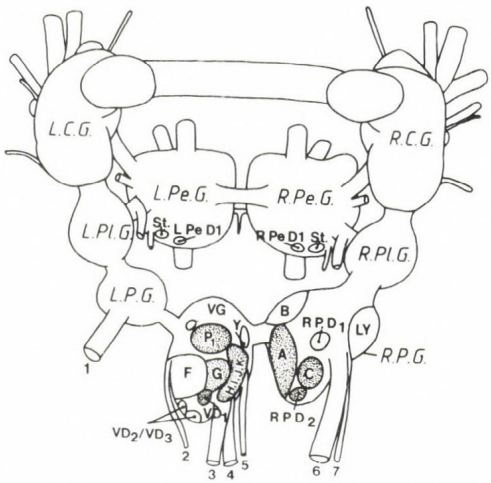

Fig. 3. Schematic representation of the dorsal view of the CNS of *Lymnaea stagnalis*, showing the position of neurons, receiving input 3 and 5 (shaded).

RPD2 and VD1

The two giant neurons, RPD2 and VD1 appear to play an important role in mediating the effects of external PO_2 changes on pneumostome movements. The two neurons are strongly electrotonically coupled resulting in 1:1 spike activity. Hyperpolarization of one of the two cells to such an extent that spike activity is suppressed, also results in suppression of spike activity in the other cell. The spike activity of both cells appears to be directly related to pneumostome movements; closure movements are accompanied by bursts of spike activity in

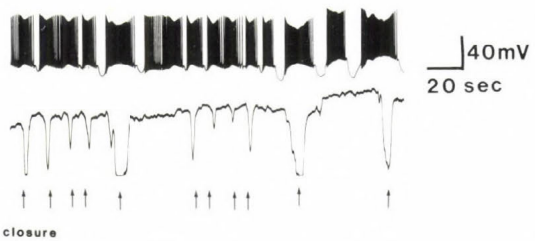

Fig. 4. Closure movements of the pneumostome (lower trace, arrows) are accompanied by bursts of spike activity in VD1 (upper trace, arrows).

VD1 and RPD2 (Fig. 4), whereas during opening movements both cells are silent (not shown).

A decrease of the external PO_2 results in a considerable decrease of spike activity in VD1 and RPD2: spike activity is almost instantaneously abolished as soon as the external PO_2 is decreased (Fig. 5). Concomitantly, the frequency of closure movements of the pneumostome decreases while the frequency of opening movements increases.

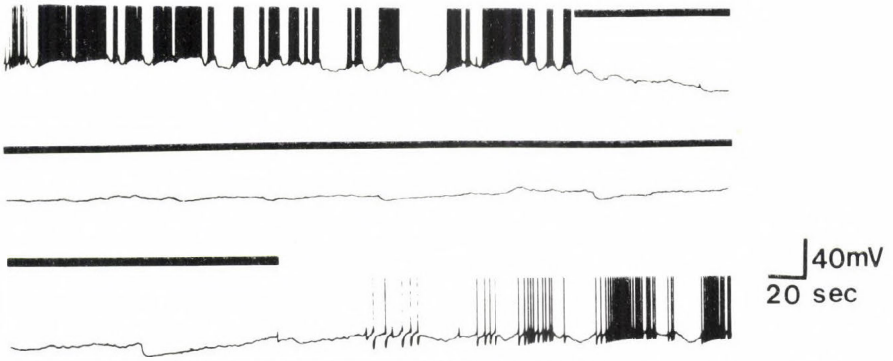

Fig. 5. Exposure of the lung/mantle area to N_2 (indicated by bar) causes hyperpolarization and complete suppression of spiking activity in VD1. Continuous recordings.

When VD1 and RPD2 are functionally eliminated from the network by applying hyperpolarizing current, the frequency and amplitude of closure movements decrease, while the frequency of opening movements increases (Fig. 6). The intimate relationship between pneumostome movements and spiking activity in VD1 and RPD2 is probably achieved by excitatory effects of input 3 and inhibitory effects of input 5 (Fig. 7). Although the origin of both inputs is unknown, their activity can be monitored by recording one or more of their follower cells. Thus, simultaneous recording of RPD2 or VD1 and a cell from the A-cluster reveals common excitatory effects, due to input 3. Hyperpolarization of VD1 and RPD2 for prolonged periods of time suppresses spiking activity completely and results in a gradual decrease of the activity

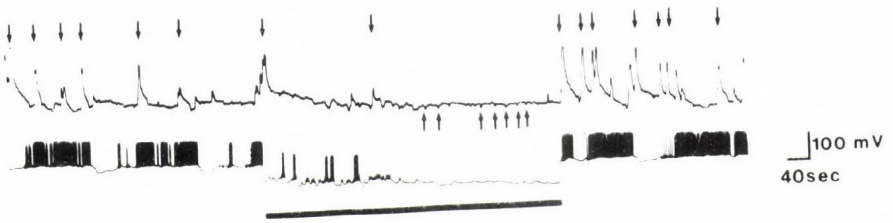

Fig. 6. Hyperpolarization of VD1 (lower trace, bar) causes a decrease in frequency of closure movements (upper trace, downward arrows) and an increase of opening movements of the pneumostome (upper trace, upward arrows). Applying hyperpolarizing current does not immediately result in complete suppression of spike activity.

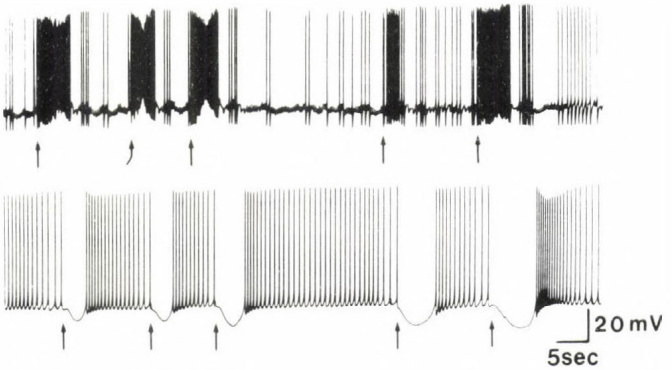

Fig. 7. Input 5 causes excitation of a P1 neuron (upper trace, arrows) and inhibition of VD1 (lower trace, arrows). Probably due to intrinsic properties of VD1, the actual hyperpolarization of this neuron is slightly delayed as compared to the occurrence of depolarization of the P1 neuron.

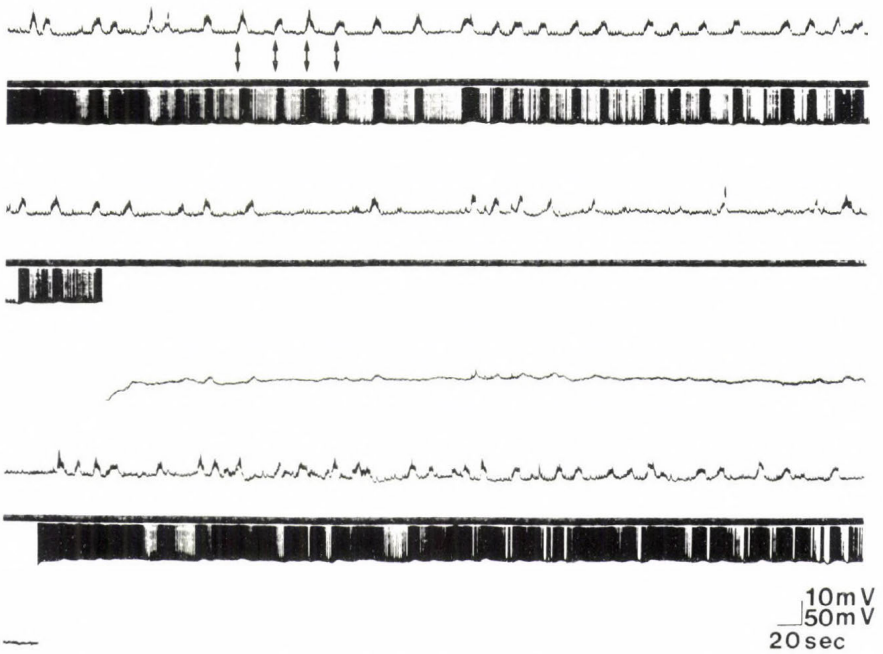

Fig. 8.Simultaneous recording of VD1 (lower trace) and a cell of the A-group (hyperpolarized to suppress spike activity) in isolated CNS. Input 3 causes depolarization of the A-cell and increased firing frequency in VD1. A number of occurrences of this common input have been indicated by arrows. Prolonged hyperpolarization of VD1 results in a decrease of the activity of input 3, as can be deduced from the decrease in frequency of depolarizations in the A-cell. Continuous recordings.

of input 3 (Fig. 8). Simultaneous recording of VD1 or RPD2 and a cell from the P1 cluster reveals that these neurons receive input 5 in common. This input exerts inhibitory effects on RPD2 and VD1 and excitatory effects on cells of the P1 cluster (Fig. 7). Prolonged hyperpolarization of VD1 results in increased bursts of activity in cells of the P1 cluster, due to an increased activity of input 5 (Fig. 9).

Fig. 9. Prolonged hyperpolarization of VD1 (upper trace) causes an increased frequency of bursts of spiking activity in a P1 neuron, due to increased activity of input 5 (indicated by bar). P1 neuron hyperpolarized; recordings in isolated CNS.

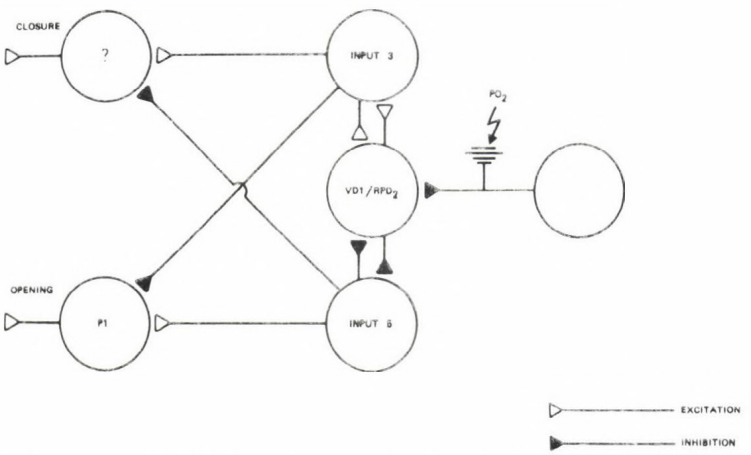

Fig. 10. Schematic representation of the main components of the neuronal network, involved in the regulation of respiratory movements. These are VD1 and RPD2, receiving O_2 chemosensory input and reciprocally connected to neurons responsible for input 3 and 5. Input 3 and 5 exert opposing effects on putative motoneurons causing opening and closure of the pneumostome. The latter are included in the network, although only circumstantial evidence suggests the presence of such neurons. For sake of simplicity, other neurons receiving input 3 and 5 are omitted. The arrangement of the network implies that the effects of O_2 chemosensory input are mediated by VD1 and RPD2, modulating the activity of input 3 and 5. As the activity of a large number of neurons is profoundly affected by input 3 and 5, changes in O_2 availability result in comprehensive changes in neuronal activity.

The onset of the effects of hyperpolarization of VD1 is rather slow and develops gradually. This suggests that the effects are not mediated by monosynaptic contacts. It is conceivable that the effects of VD1 and RPD2 on input 3 and 5 are achieved by non-synaptic release. This mode of action has been shown frequently for peptidergic cells involved in the regulation and coordination of multiple components of complex behaviour (Brown and Mayeri, 1987; Schaefer and Brownell, 1986; Mayeri *et al.*, 1979).

In summary, the contacts between VD1 and RPD2 on the one hand, and the neurons from which input 3 and 5 originate on the other appear to be reciprocal. The contacts with input 3 are excitatory, the contacts with input 5 are inhibitory. The spiking activity of VD1 and RPD2 is strongly affected by the external PO_2. A decrease of the external PO_2 results in strong inhibition of spiking activity in VD1 and RPD2. In the abscence of this input, a mutual excitation occurs between VD1 and RPD2 and input 3. When, by activation of external O_2 receptors, the activity of RPD2 and VD1 is inhibited, this reciprocal excitation is abolished. Concomitantly, the inhibitory action of RPD2 and VD1 on input 5 is removed, resulting in increased activity of these cells and subsequent additional inhibition of RPD2 and VD1. Clearly, an additional mechanism should exist to terminate the mutual inhibition by VD1 and RPD2 on the one hand and input 5 on the other. The nature of this mechanism is as yet unknown. A schematic representation of the network, discussed above, is shown in Fig. 10.

Central O_2 chemosensitivity

The effects of a decrease of the PO_2 of the saline surrounding the CNS are essentially similar to those resulting from a decrease of external PO_2. The electrical activity of RPD2 and VD1 is strongly inhibited, resulting in nearly complete suppression of spikes. The onset of the response is, however, much slower and the effect outlasts the actual change of PO_2, continuing after normoxic conditions are restored. Whether these effects are mediated by centrally located O_2 chemosensory structures, or are due to direct effects of hypoxia on the spiking activity of these cells is as yet unknown.

CONCLUSION

The effects of internal and external PO_2 changes on pneumostome movements can de explained to a large extent by the properties and connections of VD1 and RPD2. The reciprocal connections of these cells with input 3 and 5, and the O_2 chemosensory input which they receive are probably the most important aspects of the neuronal network, involved in the regulation of pneumostome movements. The effects of RPD2 and VD1 are possibly achieved by non-synaptic release of an ACTH-like peptide. By modulating the activity of input 3 and 5 the electrical activity of a large number of follower neurons in the visceral and right parietal

ganglia is affected, including a cluster of putative motoneurons. Thus, with respect to the present network, two states of neuronal activity may be distinguished. One occurs when O_2 availability is sufficient, and involves spiking activity of VD1 and RPD2, activity of input 3 and those neurons, which receive excitatory input from input 3 and inhibitory input from input 5. The other occurs when O_2 availability is low, VD1 and RPD2 are silent, input 3 is inactive, while input 5 is active, resulting in increased spiking activity of those neurons, which receive excitatory input from input 5 and inhibitory input from input 3. The reciprocal connections between VD1 and RPD2 on the one hand and neurons responsible for input 3 and 5 on the other probably result in long lasting maintenance of one of the two neuronal states. It is conceivable that in this way a coordinated, comprehensive change of neuronal activity is achieved. This is to be expected, as a change in the availability of O_2 affects a large number of behavioural and physiological processes.

ACKNOWLEDGEMENTS

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LOCOMOTION IN LYMNAEA – ROLE OF SEROTONERGIC MOTONEURONES CONTROLLING THE PEDAL CILIA

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INTRODUCTION

The existence of ciliated cells is well documented throughout the animal kingdom (Eckert, 1983). Their importance in the mammalian reproductive and respiratory systems is well known, whilst in aquatic pulmonate molluscs such as **Lymnaea stagnalis**, ciliary beating produces the primary locomotor force (Jones, 1975). "Steering" movements and twisting movements of the foot are brought about by muscular contractions of the foot and body wall. Rhythmic shell movements are usually associated with locomotion (Winlow & Haydon, 1986), but may be part of the respiratory and/or cardiovascular pumping mechanisms of the animal.

Behaviour in gastropod molluscs is often organised on a multiganglionic basis and individual ganglia are often much less autonomous than those of insects (McCrohan & Winlow, 1985). Here we demonstrate that while the serotonergic motoneurones controlling the pedal cilia of **Lymnaea stagnalis** are found in the pedal ganglia, they form part of a major integrative network. This network acts as a neural substrate for locomotion itself; feeding; shell movements associated with respiration, whole animal withdrawal responses and egg-laying behaviour.

CILIA OF THE PEDAL EPIDERMIS

Scanning electron-microscopy reveals that the sole of the foot of **Lymnaea** is covered with a dense carpet of cilia (Fig. 1) and electron microscopy indicates that the bulk of the pedal epidermis is made up of ciliated cells, broadly similar in ultrastructure to those found in a wide range of other invertebrates (Bereiter-Hahn et al., 1984; McKenzie et al., 1987). Ciliated cells are believed to be innervated by serotonin-containing axon terminals (see Caunce et al., this volume).

Fig. 1. A scanning electron-micrograph of the pedal cilia of **Lymnaea.**

MORPHOLOGY OF CILIOMOTONEURONES

a) **Location.** The paired symmetrical pedal A-cluster neurones of **Lymnaea stagnalis** are located on the medial faces of the largely identified right and left pedal ganglia. There are 30 individually identifiable neurones in each cluster as shown in Fig. 2. The cells are numbered from posterior to anterior in the sequence 1 to 30. Cells 1-12 lie on the dorsal surfaces whilst 13-30 lie on the ventral surfaces of the ganglia. Preliminary studies with semi-intact preparations demonstrate that intracellular stimulation of neurones of the pedal A-clusters elicits excitation of the pedal cilia and increases mucus production from mucus-containing cells of the foot.

b) **Somatotopic organisation of ciliomotoneurones.** By use of a modified technique (Syed & Winlow, in preparation) for injection of the neural marker substance, Lucifer Yellow CH (Stewart, 1978) we have clearly demonstrated that neurones of the pedal A-clusters are somatotopically organised (Fig. 3). Neurones at the anterior end of the clusters exit via the posterior nerve trunks and **vice versa**, whilst those in the centre of the cluster project down medial pedal nerve trunks. Anteriorly and posteriorly located nerve trunks project to the anterior and posterior areas of the foot sole respectively. Neurones of the adjacent B- and E-clusters are quite different in structure and cannot be mistaken for A-cluster neurones.

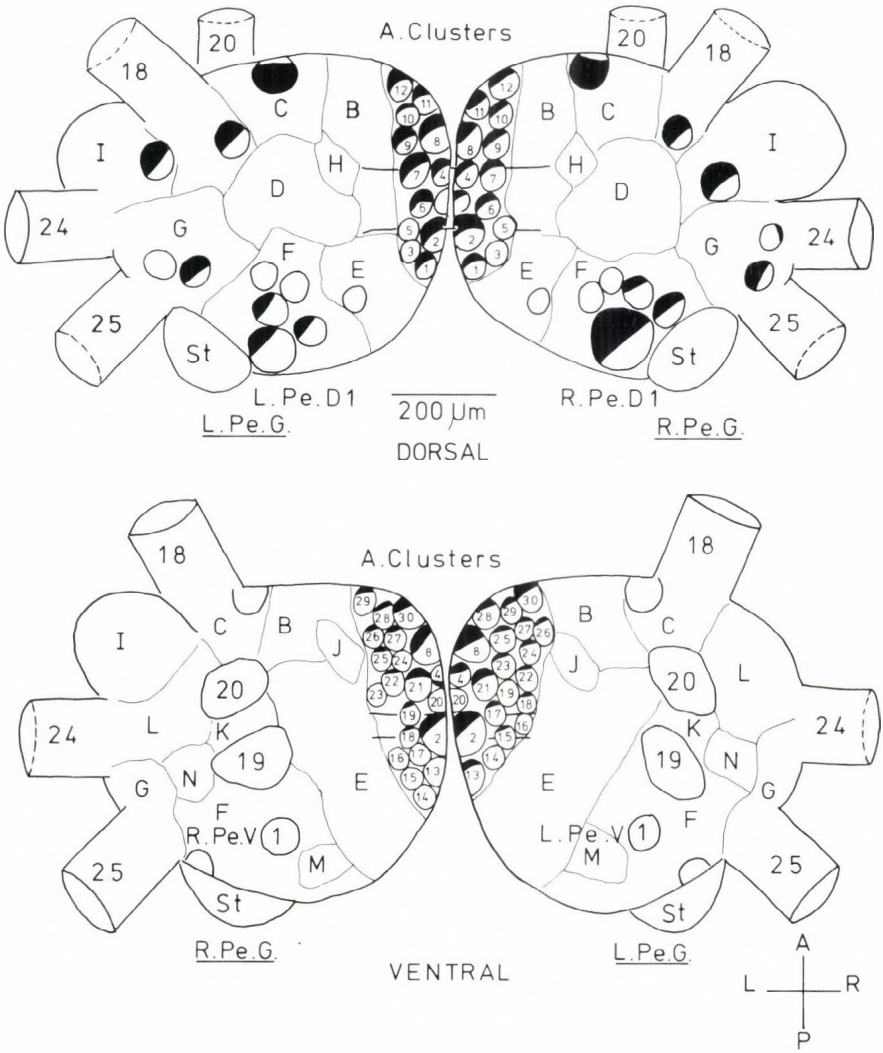


Fig. 2. Location of individually identifiable pedal A-cluster neurones on the medial faces of the pedal ganglia. Diagrams modified from Slade et al.(1981). The pairs of giant neurones originally identified within the clusters as L./R.Pe.V.2; L./R.Pe.D.4 and L./R.Pe.D.8 are now considered to be an integral part of each cluster and have been renamed L./R.Pe.A2, L./R.Pe.A4 and L./R.Pe.A8 respectively.

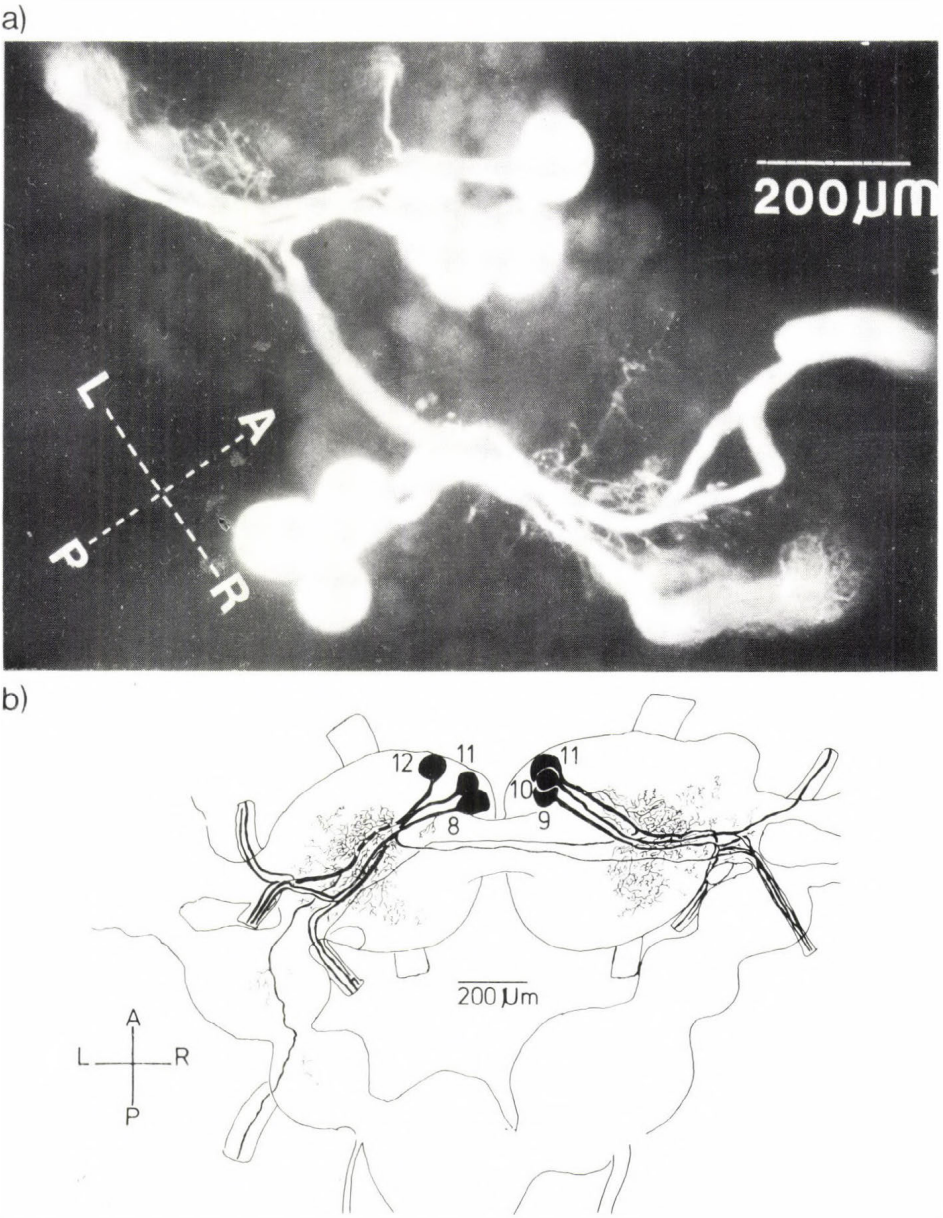


Fig. 3. Somatotopic organisation of ciliomotoneurones.
a) Photomicrograph of ciliomotoneurones showing their somatotopic organisation. Anterior neurones exit via the posterior nerve trunks and **vice versa.**
b) **Camera lucida** drawing of ciliomotoneurones. Note the contralateral projections of L.Pe.A8 and R.Pe.A11.

CILIOMOTONEURONES ARE SEROTONERGIC

By use of the glyoxylic acid condensation technique (de la Torre & Surgeon, 1976), it has recently been demonstrated that the cell bodies of the ciliomotoneurones contain serotonin (Casey & Winlow, 1985; Audesirk, 1985). Using an HPLC microassay for serotonin developed by Dr. Dean, Department of Pharmacology, Leeds, lumped samples of A-cluster neurones were found to contain 1-2 μg of serotonin per cell. Further studies of these neurones using immuno-fluorescent probes are in progress. Finally we have demonstrated that injection of serotonin into the foot causes an increase in the rate of ciliary beating as measured by the rate of particle transport in intact animals (Fig. 4). Experiments with serotonin antagonists on the isolated foot are in progress.

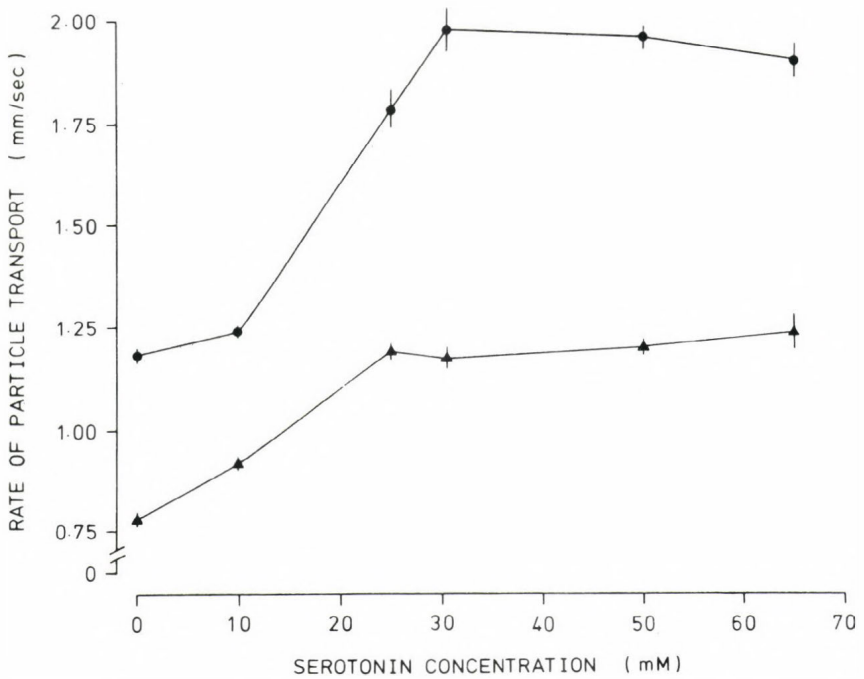


Fig. 4. The rate of particle transport (iron filings) across pedal epithelium increases with increasing concentrations of serotonin injected into the foot. The rate of particle transport in air (▲) is rather lower than when the animal is submerged(●). For these experiments the animals were inverted on a piece of "Plasticine" in a small experimental chamber. Vertical lines indicate standard errors of the mean.

PHYSIOLOGY OF CILIOMOTONEURONES

a) **Electrical connections.** The majority of the ciliomotoneurones project ipsilaterally and are weakly electrically coupled to their near neighbours. The larger cells in the clusters project both ipsilaterally and contralaterally and are electrically coupled to their contralateral homologues as well as to their smaller neighbours (Fig. 5a) that only project ipsilaterally.

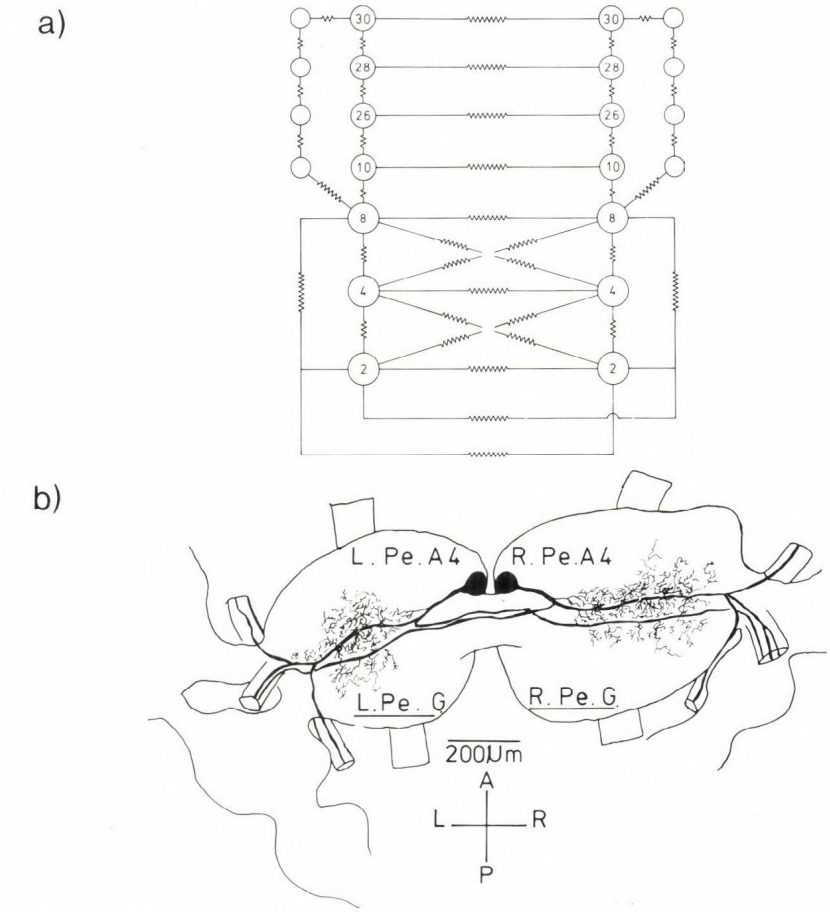


Fig. 5 Electrical coupling between ciliomotoneurones.
a) Summary diagram. The giant neurones A2, A4 and A8 are coupled to their contralateral homologues, to one another and to the smaller cells surrounding them. Several smaller cells A10, A26, A28 and A30 have similar characteristics. The remainder are connected only to their near neighbours and not to their contralateral homologues.
b) **Camera lucida** drawing of the projections of the contralaterally coupled left and right pedal A4 neurones (formerly L./R.Pe.D.4).

b) **Common synaptic inputs from unidentified sources.** The ciliomotoneurones receive a number of common synaptic inputs. All the cells in the paired A-clusters receive a regular excitatory synaptic input which we have termed the "ciliary rhythm" so called because of its presumed involvement in ciliary locomotion (Fig. 6) since it is not received by any other neurones. In addition they receive input 4 (Winlow & Benjamin, 1976, Winlow, 1979), a wide-acting identified synaptic input which also drives neurones of the visceral and right parietal ganglia (Fig. 7). Input 3, (Benjamin & Winlow, 1981) which we believe is involved in respiration and which has powerful effects on the follower cells of the giant dopamine containing neurone R.Pe.D.1; also has widespread effects on the paired neurones of the pedal ganglia. The ciliomotoneurones receive inhibition immediately following the effects of input 3 on other neurones (Fig. 8).

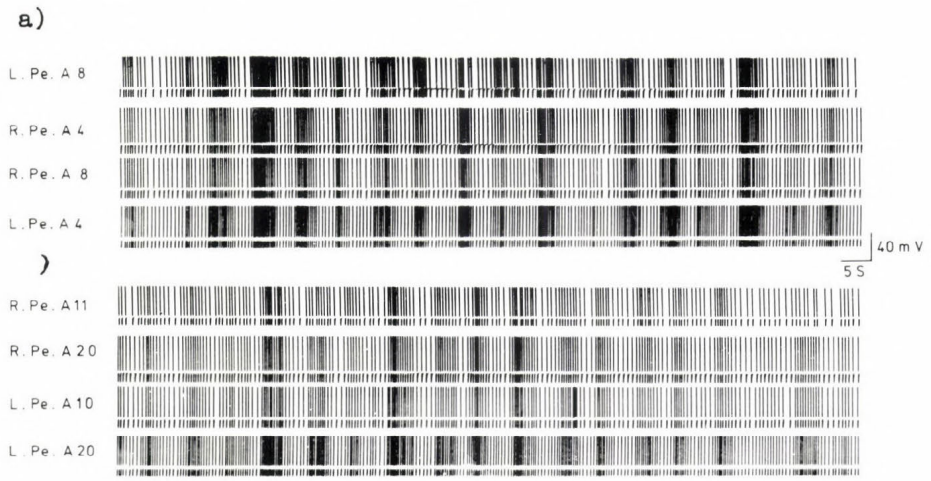


Fig. 6. Examples of the "ciliary rhythm" received only by ciliomotoneurones. The rhythm is recorded in a) L./R.Pe.A4 and L./R.Pe.A8 cells and b) from L.Pe.A10; R.Pe.A11 and L./R.Pe.A20.

c) **Synaptic inputs from identified cells.** The "ring neurone" is an interneurone which lies in the right cerebral ganglion and forms a loop via the pedal ganglia and back into the left cerebral ganglion (Jansen & Maat, 1985). It excites the caudodorsal cells (CDCs), which are involved in egg-laying behaviour (Jansen & Bos, 1984), and various locomotor motoneurones of the pedal ganglia involved in muscular

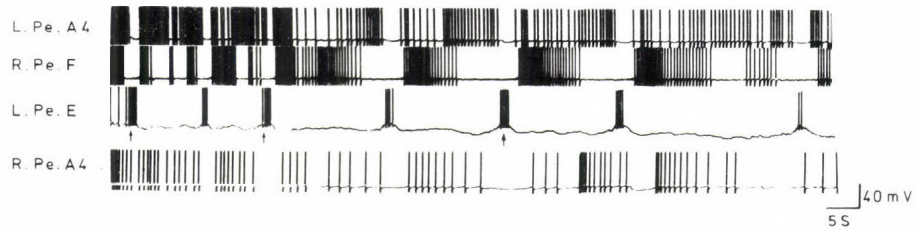


Fig. 7. The actions of wide-acting input 4 (arrows) on ciliomotoneurones (L./R.Pe.A inhibition) and other locomotor motoneurones (R.Pe.F. inhibition, L.Pe.F. inhibition, L.Pe.E excitation).

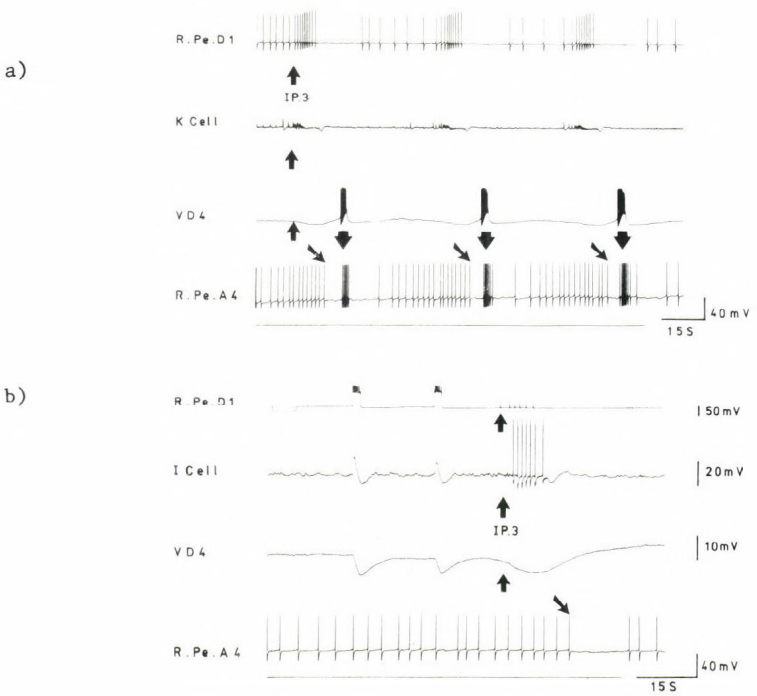


Fig. 8. The actions of wide acting input 3 on various neurones.
a) Delayed inhibition of ciliomotoneurones following the action of input 3 on other pedal and visceral neurones in the isolated brain. VD4, which is inhibited by input 3, fires immediately following input 3 and as a result either excites or inhibits its own follower cells.
b) Identified interneurone R.Pe.D.1(top trace) has biphasic effects (excitation followed by inhibition) on an I cell, inhibits VD4 and switches on input 3 (↑) by post-inhibitory rebound excitation (Winlow et al., 1981). Thus input 3 has delayed inhibitory effects on A-clusters.

movements of the foot. In addition it inhibits the ciliomotoneurones (Jansen & Maat, 1985; Syed & Winlow, in preparation).

Another wide-acting interneurone is known as either VD4 (Janse et al., 1985) or the visceral white interneurone (Benjamin, 1984). It has widespread effects on neurosecretory neurones as well as an overall inhibitory effect on another interneurone R.Pe.D.1 which itself reciprocally inhibits VD4. These connections appear to us to be monosynaptic. The actions of VD4 on the follower cells of R.Pe.D.1 are opposite to one another in most cases, e.g. R.Pe.D.1 inhibits the visceral J. cells and excites the K cells (Winlow et al., 1981) whilst VD4 has the opposite actions on these neurones (Syed & Winlow in preparation). In addition both R.Pe.D.1 and VD4 are able to induce the onset of input 3. VD4 has widespread actions on motoneurones of the pedal ganglia (Fig. 9a & b) and excites the ciliomotoneurones (Fig. 9b).

Ciliomotoneurones also receive excitatory inputs in common with other locomotor motor neurones during both terresterial and aquatic locomotion recorded from semi-intact preparations. This activity also occurs in isolated brains (Fig. 10) and suggests that the central pattern generator for locomotion resides within the central nervous system and is capable of driving locomotor activity without the aid of peripheral inputs.

CONCLUSIONS

Despite the fact that all the A-cluster neurones project deep into the foot they do not cause contraction of the foot, body wall or column. Nevertheless, they do receive excitatory inputs during retraction and protraction phases (Winlow & Haydon, 1986) of terrestrial locomotion and during column contraction, when the shell is pulled onto the animal. Electrical stimulation of a large number of these serotonergic neurones excites previously quiescent cilia, with a latency of a few seconds and they remain excited for several seconds once the stimulus is terminated. The low frequency discharge of pedal A-cluster neurones, as compared to the much higher ciliary beat frequency, suggests that the cilia are tonically excited by the A-cluster neurones.

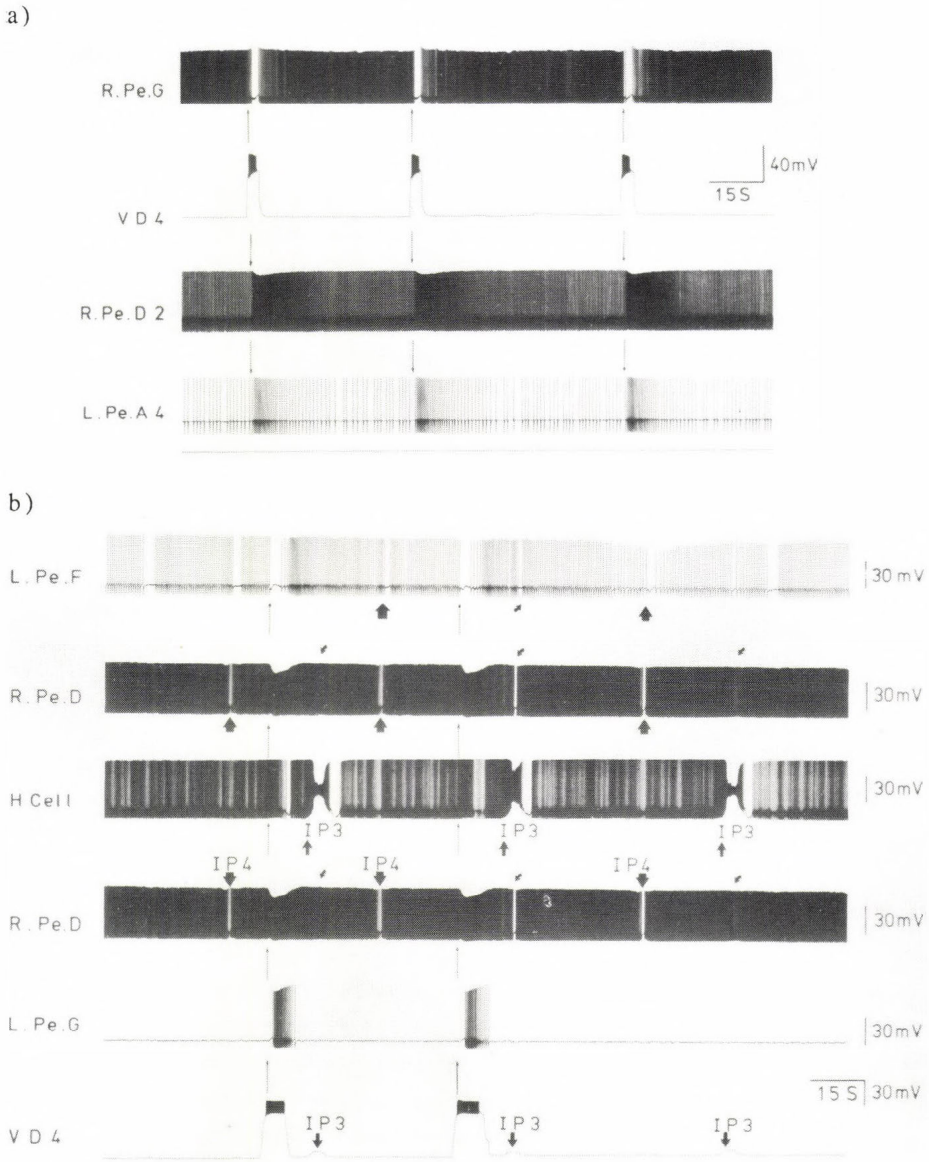
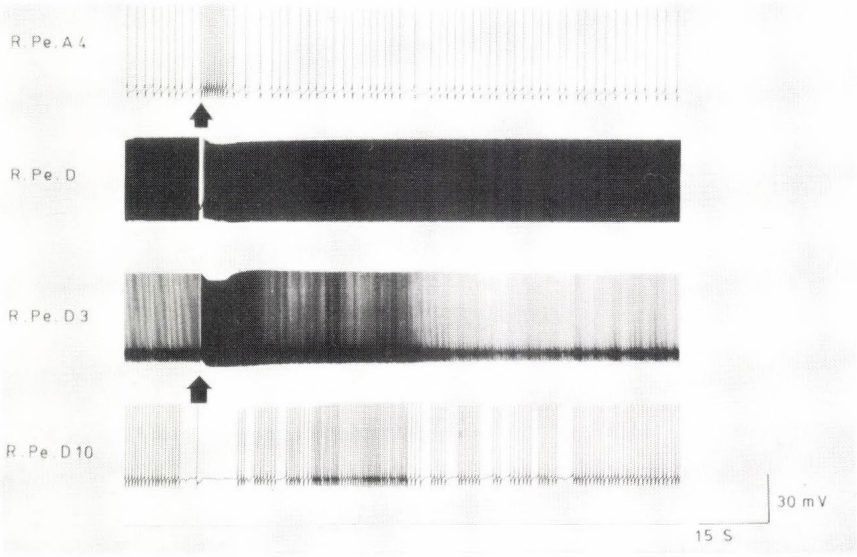


Fig. 9.

a) Connections and interactions of VD4. The action of VD4 on neurones of the pedal ganglia, as recorded from a semi-intact preparation. VD4 inhibits the R.Pe.G. cluster (top trace) and excites R.Pe.D.2 and L.Pe.A4. These effects are believed to be monosynaptic in most of the cases studied.

b) Simultaneous recordings from six different neurones showing the effects of VD4 on its follower cells. Figure also shows effects of IP3 (↶) and IP4 (↓) on pedal neurones and IP4 on visceral cells.

a)



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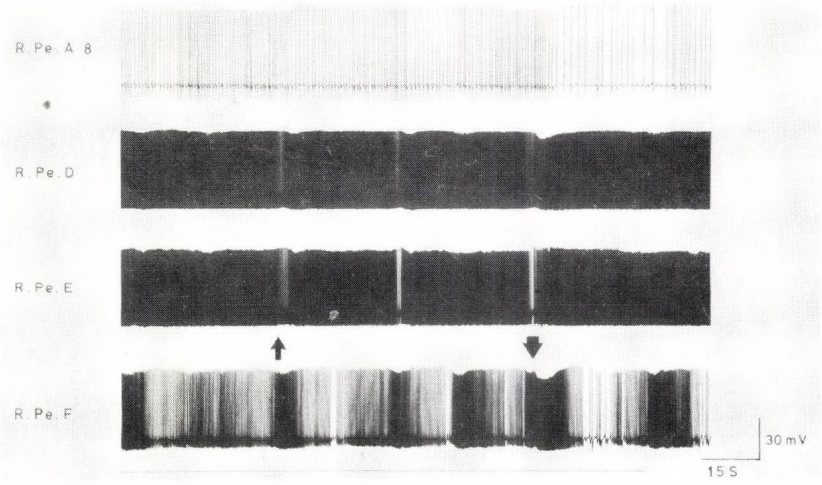

Fig. 10. Recordings of "locomotor activity" from neurones of isolated brains.
a) Inputs to ciliomotor and locomotor neurones from the central pattern generator (⇑). From our studies with semi-intact preparation we know that such inputs are received when the shell is pulled on to the animal at the end of the locomotor phase. Here they occur in the absence of sensory inputs.
b) Locomotor inputs equivalent to those occurring during terrestrial locomotion. (↑) indicates contractions of the foot whilst (↓) indicates contraction of the columellar muscle.

In previous studies, we have demonstrated the presence of serotonin in the foot, which is present in small varicosities and also know that both the ciliated cells and mucus secreting cells are innervated (McKenzie et al., in press). At present experiments are in progress at E.M. level to demonstate that the axon terminals on ciliated and/or mucus cells contain serotonin.

The available evidence does then strongly suggest that the A-cluster neurones control ciliary beating and that these serotonergic neurones are an integrated part of a major neuronal network which acts as a substrate for several complex behaviours.

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DISCUSSION

ALKON, D.L.: Could the ciliary motor system you describe be considered largely as an effector system, i.e. both the cilia and their motor control neuronal network would be accessible to a variety of sensory inputs which, depending on their intensity, might most crucially determine ciliary motor effect(s) at a given time?

SYED, N.I.: Yes, this is a very interesting comment. In our preparations we have noticed a very closely linked integration between ciliomotoneurones and locomotor motoneurones since they are both involved in the locomotor behaviour. It is probable that specific behaviours are moulded by sensory inputs of varying intensity and are drawn out of the major network which we have described as being involved in a variety of behaviours.

WINLOW, W.: Dr. Alkon made the point that only relatively small numbers of neurones exist within the snail nervous system. Thus it is necessary for groups of neurones to be used in a variety of different ways to produce different behaviours. The large network we have described is really a neuronal substrate out of which behaviours as diverse as feeding, locomotion, shell movements, egg-laying behaviour, etc., can be called down as appropriate and integrated with cardiorespiratory responses.

CARLBERG, M.: Is the glyoxylic acid method your only criterion for 5-HT?

SYED, N.I.: Fortunately not. We have applied other techniques as well, e.g. immunohistochemistry and HPLC methods, and they all give us similar answers.

JANSE, C.: Can you elaborate on how the A cells control the cilia beat? Are the ciliated cells spontaneously active and is their activity modulated by the A cells?

SYED, N.I.: Please allow me to correct your terminology first. It is A-cluster not A group or A cells. Now to your question, as I mentioned earlier pedal A-cluster neurones excite cilia in a tonic manner. We believe that serotonin is released from the terminals which are present on both ciliated cells and mucus cells, and excite cilia. Since their ciliary beat frequency is far greater than the discharge rate of A-cluster neurones, it is less likely that cilia are excited beat for beat. Our electrophysiological studies have shown that electrical stimulation of a large number of A-cluster neurones

(a) excite pedal cilia if they are quiescent;
(b) increase their frequency if they are beating already.

This probably suggests that the A-cluster neurones modulate ciliary beat frequency and are correctly termed ciliomotoneurones. Further experiments are in progress to prove this point.

LUKOWIAK, K.: You mentioned that the A group cells, especially the large ones, were electrically coupled, yet in some of your records this is difficult to see. What are the coupling ratios and/or why don't we see this coupling?

SYED, N.I.: As I showed you earlier, the A-cluster neurones are weakly electrically coupled, and this coupling is not as strong as we see in case of VD1 and RPD2 or right and left cerebral giant cells of Lymnaea. Nevertheless, a pulse of depolarizing or hyperpolarizing current applied to one cell can be recorded from its neighbouring cells for contralateral homologues, and vice versa. We have not calculated the coupling ratio. All we know is that these cells are weakly electrically coupled. However, we occasionally see de-coupling among these cells recorded from semi-intact preparations during turning behaviour.

van der WILT, G.: Could you comment on the physiological significance of the wide-distributing nature of the synaptic inputs that presently have been described in Lymnaea? Numerous neurones seem to receive synaptic input that is related to a cer-

tain type of behaviour, while a significant role of these neurones in regulation of this particular behaviour is apparently absent.

SYED, N.I.: Had you asked this question a few years ago, it would have been difficult to answer, but from our recent work with semi-intact preparations we can clearly define a specific behaviour with respect to the inputs that are observed on different cells during that particular behaviour, e.g. during feeding, locomotion is terminated and hence the locomotor motoneurones receive inhibition. Similarly during egg-laying behaviour the Ring neurone excites CDCs and inhibits ciliomotoneurones, thus terminating locomotion. On the other hand, RN excites foot and body wall motoneurones to cause the contraction of those muscles, hence facilitating egg-laying behaviour. Similarly during breathing, all these inputs are well defined on isolated brains. We have substantial evidence and this will appear in the press in the near future.

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REVERSIBLE EFFECT OF 5,6-DHT TREATMENT ON HELIX: A COMBINED BEHAVIOURAL, ELECTROPHYSIOLOGICAL AND BIOCHEMICAL STUDY

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INTRODUCTION

In the molluscan nervous system serotonin was found to be a neurotransmitter involved in synaptic transmission (Cottrell and Macon, 1974; Gerschenfeld and Paupardin-Tritsch, 1974). Moreover serotonin has a modulatory effect on various behaviours such as feeding, circulation or some forms of learning (Weiss et al., 1978; Liebeswar et al., 1975; Hawkins, 1984; Crow and Forrester, 1986).

The toxic analogues of serotonin: 5,6- and 5,7-dihydroxytryptamines (5,6-DHT and 5,7-DHT) are widely used in studies related to serotonergic neuronal systems (Hiripi et al., 1981; Glover and Kramer, 1982; Baumgarten et al., 1982), thus the functional consequences of the treatment may reflect the role of serotonin in normal state.

In the CNS of Helix after dihydroxytryptamine treatment a brown pigmentation develops in the somata of a particular population of neurons which are suggested to be serotonergic (Pentreath and Berry, 1978; Balaban et al., 1985; S.-Rózsa et al., 1986). These studies also reported that the pigment containing cells displayed their normal electrophysiological characteristics and inputs as well.

In order to use the dihydroxytryptamine induced brown pigmentation as a marker of the serotonergic neurons, on which normal electrophysiological experiments can be performed the reversible effect of this neurotoxin had to be demonstrated.

In the present study the behavioural and electrophysiological consequences of 5,6-DHT injection were investigated. Parallel with these the changes of the serotonin content were detected in the central ganglia.

MATERIAL AND METHODS

Adult specimens of Helix pomatia (collected locally) received 5,6-DHT (5,6-dihydroxytryptamine creatinine sulphate from SIGMA, dissolved in snail saline) injection of 20 mg/kg body weight and 50 µl volume per individual into the body cavity. The drug solution contained 0.5 mg/ml ascorbic acid as antioxidant.

(a) To study the behavioural changes after the treatment the build-up of the feeding arousal state was tested, which is reported to be serotonin dependent on both Aplysia (Kupfermann and Weiss, 1982) and Helix (Kemenes and S.-Rózsa, 1987).

The freely moving animals were tested individually on large pieces of filter paper soaked with 3% solution of sucrose which had proved to be an effective and standardizable feeding stimulus (Kemenes et al., 1982). The snails were placed on filter paper and the time between the contact and the first bite carried out by the animal was measured. Each animal was tested 25 times with a 20 s interval between the end of the previous test and the beginning of the next one.

(b) The effectiveness of the serotonergic synaptic transmission was tested on the identified synaptic connections (Cottrell and Macon, 1974) between the giant serotonergic cell (GSC) located in the cerebral ganglia and its followers "A" and "M", respectively, both of them located in the buccal ganglia. Electrophysiological experiments were carried out on isolated CNS (ganglionic ring plus buccal ganglia) preparations by conventional electrophysiological methods.

(c) For biochemical studies different tissues, i.e. paired cerebral ganglia, paired buccal ganglia, suboesophageal complex and buccal mass, were dissected and homogenized separately from vehicle-injected (control) or drug-treated animals. The

serotonin and dopamine contents of these samples were determined with HPLC method (Saller and Salama, 1984).

RESULTS

(a) The feeding arousal state can be characterized by the feeding latency which is the time duration between the food presentation and the beginning of the feeding behaviour. The shortening of this latency during consecutive presentations of food reflects the build-up of the feeding arousal state.

Before injection, specimens of both groups reached a similar level of feeding arousal state of the same latency (Fig. 1).

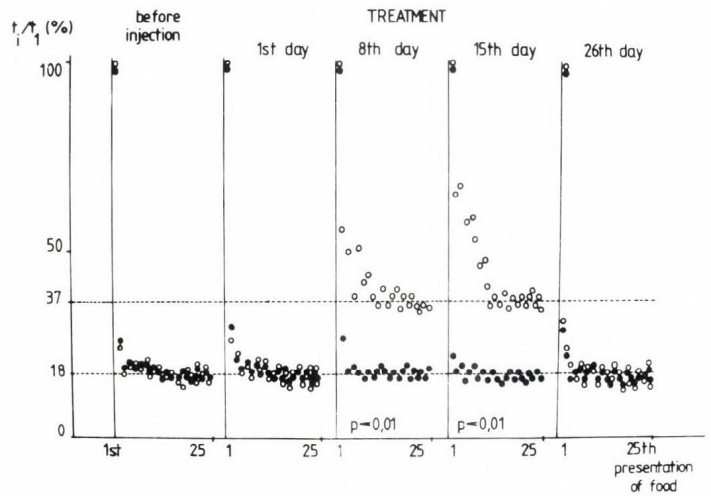

Fig. 1. Build-up of the feeding arousal state in control and 5,6-DHT treated animals. t_1 represents the very first feeding latency as 100 per cent and t_i represents the latency of the following presentations of food. The scores represented by ● (control animals, vehicle injected) and o (5,6-DHT injected snails) were calculated as percentage values with the very first feeding latency (t_1) as 100 per cent.

No differences were observed between the two groups 1-5 days after the injection. Testing the animals on the 8th day following 5,6-DHT treatment, the build-up of the feeding arousal state reached its baseline value more slowly in the injected

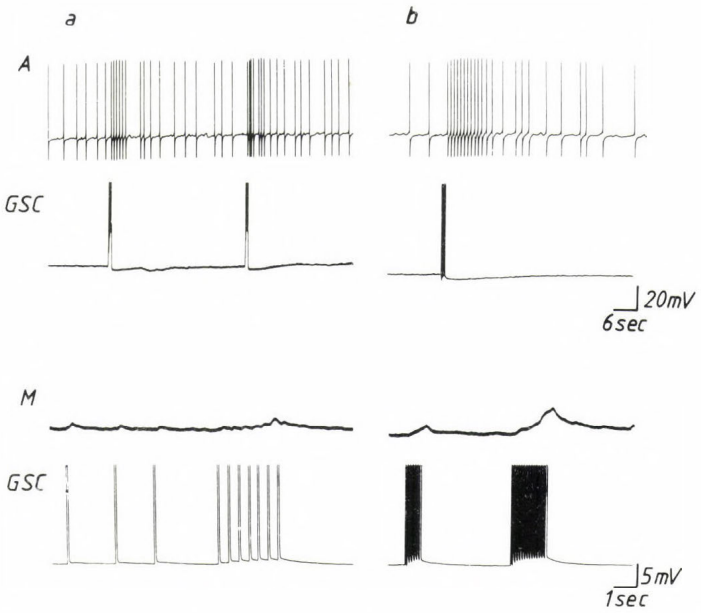

Fig. 2. Identified serotonergic synaptic connections between the giant serotonergic cell (GSC) and its followers. 1. Action potentials evoked by depolarizing current (a) or train of pulses (b) on GSC excite the neuron "A" increasing the spontaneous activity of the cell. 2. Depolarizing pulses applied to the GSC evoke single excitatory postsynaptic potential (a) or summated EPSPs (b) on "M", when the membrane is hyperpolarized to -120 mV.

animals, moreover the members of the 5,6-DHT treated group reached a higher baseline value of the feeding latency, representing a lower level of the feeding arousal state.

After the 26th day of 5,6-DHT treatment the animals showed normal feeding behaviour again; all specimens of both groups reached similar values of the feeding latency with similar dynamics in a series of food presentation.

(b) In autumn, when our studies were carried out, the synaptic connections between the giant serotonergic cells (GSCs) of the cerebral ganglia and their followers in the buccal ganglia could be recorded reliably in control and vehicle-treated preparations (Fig. 2). Depolarization of the GSC led to increasing spontaneous activity of buccal neuron "A", while buccal neuron "M" displayed monosynaptic excitation with post-

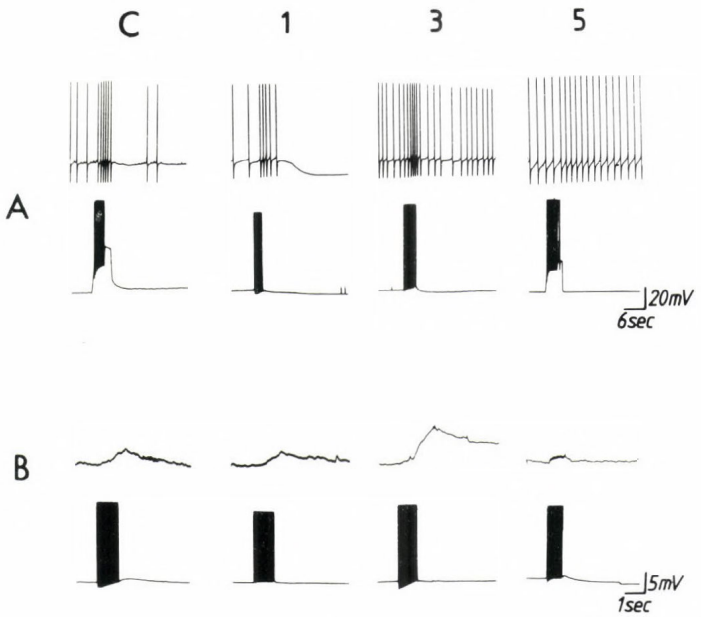

Fig. 3. Changes of the serotonergic synaptic transmission after 5,6-DHT treatment I. On the 1st-3rd days after 5,6-DHT injection the synaptic transmission between the GSC (lower trace in A and B) and the buccal cell (A: upper trace) as well as the neuron "M" (B: upper trace) seemed to be intact but by the 5th day both of them became less effective. C: recording from control preparation made from untreated animal.

synaptic potentials (EPSPs) following one for one action potentials (APs) in the GSC. One to three days after 5,6-DHT treatment the synaptic connections between the identified cells were still intact, but after the 5th day of injection the transmission between the GSC and the buccal cell "A" as well as the buccal cell "M" became less effective (Fig. 3).

Between the 8th-15th days of treatment the synaptic connection was blocked on preparations made from neurotoxin injected animals, and by the 21st day the restoration of both the synaptic connections (e.g. excitatory action of GSCs on both followers) could be observed again (Fig. 4).

(c) The paired cerebral ganglia of Helix contain GSCs, thus the 5HT level of these tissues represents the serotonin content mainly of the somata of these neurons. The suboesophageal ganglionic complex includes cell bodies and axonal processes of

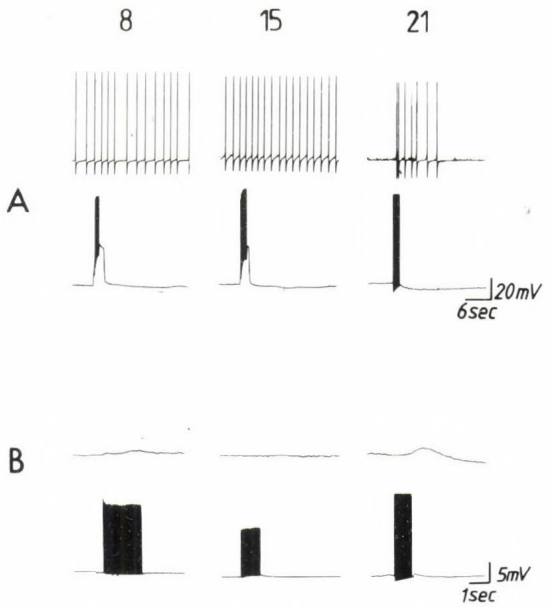

Fig. 4. Changes of the serotonergic synaptic transmission after 5,6-DHT treatment II. On the 8th-15th days of the 5,6-DHT treatment the synaptic transmission between the GSC and the buccal cell "A" (A) and the buccal cell "M" (B) was impaired, but by the 21st day a restoration of both connections was recorded.

several serotonergic neurons situated in the paired pedal, visceral and right parietal ganglia.

Between the 1st and 3rd days after treatment the 5HT content decreased in both cerebral ganglia and this decreased level of transmitter could be observed up to the 22nd-24th days (Fig. 5).

In the buccal ganglia and buccal mass, containing serotonergic axonal terminals without cell bodies, the 5HT level reached its minimum value on the 3rd day of treatment while the restoration of the 5HT content was detected from the 22nd-24th days (Fig. 6).

In preparations made from vehicle-treated animals a smaller decrease of the 5HT content was also observed mainly in the suboesophageal complex and buccal mass.

The presence of the injected 5,6-DHT was also detected in the same tissues; after an initial, exponential drop the neuro-

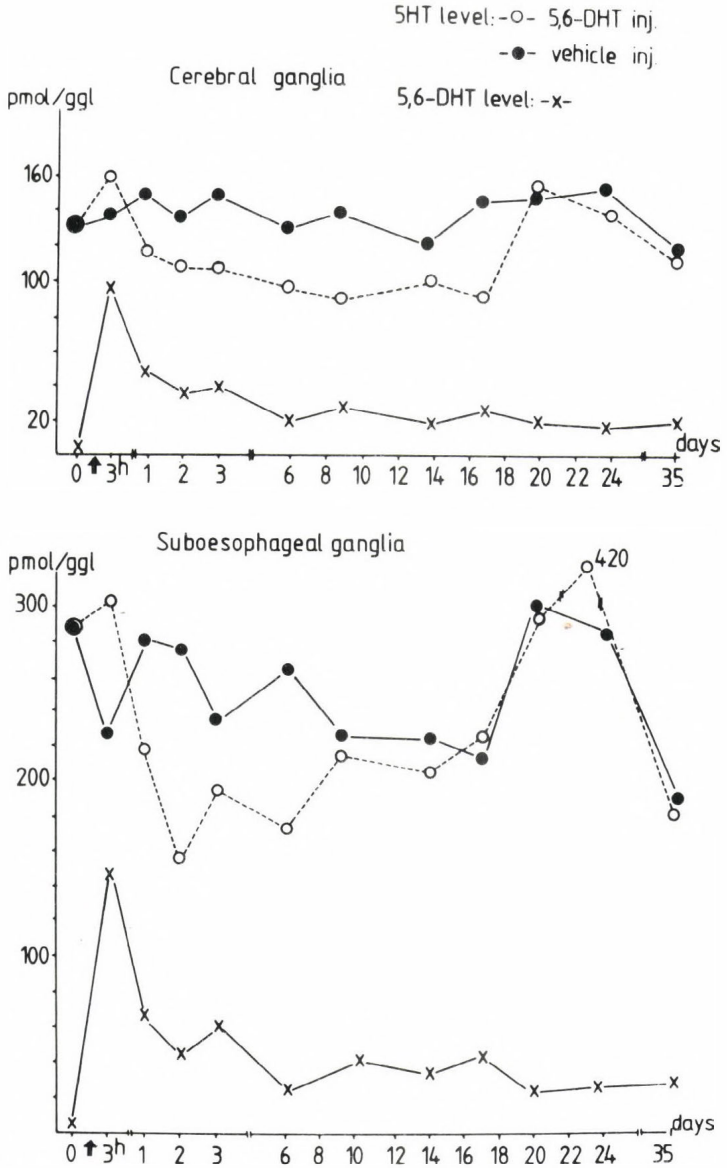

Fig. 5. Serotonin content of cerebral and suboesophageal ganglia after 5,6-DHT injection (arrow) detected by HPLC assays.

toxin was stabilized at constant level in each sample, indicating a long-lasting, non-specific binding to proteins.

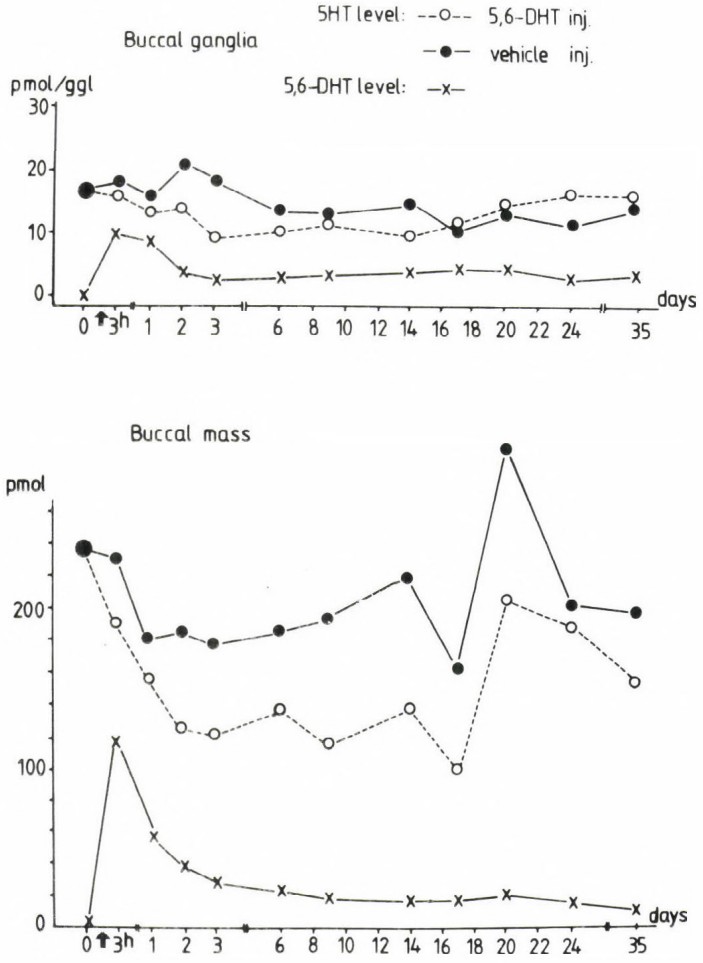

Fig. 6. Serotonin content of the buccal ganglia and buccal mass after 5,6-DHT treatment (arrow) assayed by HPLC method.

DISCUSSION AND CONCLUSION

The 5,6-DHT treatment caused transient and reversible suppression of the investigated serotonin-dependent functions.

On the 3rd-5th days of treatment biochemical analysis showed a depression of the 5HT content in all tissues investigated but the serotonergic functions (e.g. serotonergic synaptic trans-

mission and the build-up of the feeding arousal state) seemed to be intact. Differences between the results obtained by biochemical analysis and electrophysiological studies as well as behavioural experiments may reflect some compensatory mechanism taking part in the first days of neurotoxin treatment.

By the 5th-8th days the 5HT level of the neuronal tissues reached a lower, nearly constant level, and parallel with this the effectiveness of the synaptic transmission as well as the serotonin dependent part of the feeding behaviour became impaired.

From the 22nd-24th days of 5,6-DHT treatment no differences were observed between the control and experimental animals either at behavioural level or in synaptic transmission. By this time the serotonin content of the tissues assayed by HPLC reached the control level.

Summarizing our results we may conclude that 5,6-DHT treatment proved to be reversible, thus the 5,6-DHT-induced brown pigment-containing neurones can be used in physiological experiments in the same way as that of the neurones of untreated animals.

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DISCUSSION

ALKON, D.L.: Would it not be more accurate to describe synaptic connections between neurons as monosynaptic only after having satisfied a number of physiologic and, preferably, anatomic criteria? Such considerations as brief latency, one-for-one following of synaptic potentials after pre-synaptic impulses, graded control of synaptic potential by pre-synaptic polarization, etc. might be included.

SCHMIDT, E.D.: I have a comment on that. I would suggest to add to the two mentioned types (mono- and polysynaptic) the third possible type of interneuronal communication: I refer to non-synaptic communication (communication between neurons), without the necessity of having structural contacts, via neurochemical messengers released into the intercellular space of the CNS.

SYED, N.I.: Had you regulated the temperature during your experiments, because we have noticed that slight changes in the temperature affect serotonin contents significantly?

VEHOVSZKY, Á.: We did not regulate the temperature during our experiment; but the parallel fluctuations observed between the drug-treated and vehicle-injected preparations may reflect the influence of temperature changes during this period.

WINLOW, W.: As to N.I. Syed's question, using HPLC techniques we have recently found, in preliminary studies, that a temperature drop of as little as 2-3^{o}C ("cold shock") can markedly in-

crease the concentration of serotonin in the brain in general and the pedal A cluster cells in particular.

WALKER, R.J.: I was very interested in the observation that while pretreatment with 5,6-diOH tryptamine only reduced the serotonin content of the cerebral ganglia to about 70% of control, the monosynaptic potential in the buccal follower cell following cerebral giant serotonin cell stimulation was completely absent. I thought that the level of a transmitter would have to be reduced to a far greater extent before the synaptic event would be blocked. What was the effect of iontophoretic application of 5HT on the follower cells following pretreatment with 5,6-diOH tryptamine?

VEHOVSZKY, Á.: In these series of experiments we did not use iontophoretic application of 5HT on the follower cells.

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5,6-DIHYDROXYTRYPTAMINE-INDUCED CHANGES IN THE SEROTONERGIC MODULATION OF FEEDING IN LYMNAEA

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INTRODUCTION

The serotonin-containing Cerebral Giant Cells (CGCs) of the snail Lymnaea were previously shown to have a modulatory role in the feeding circuitry (McCrohan and Benjamin, 1980b). Feeding motoneurones were excited by the CGCs via mono- and polysynaptic connections and this increased the intensity of motoneurone firing in the isolated ganglion preparation. However, the CGCs also had synaptic connections with the pre-motor neuronal circuitry (central pattern generator) responsible for rhythm generation as well as certain modulatory neurones such as the Slow Oscillator (SO) (Benjamin et al. 1981). This suggests that the CGCs have a more complex function than just increasing the strength of muscular contractions in the buccal mass. One possibility is that they play a similar role as the homologous metacerebral giant cells occurring in Aplysia which are concerned with food-induced arousal (Weiss et al., 1981). In Aplysia this probably involves both the central and peripheral release of serotonin (5-HT) and this is compatible with the known anatomy and physiology of the CGCs in Lymnaea (McCrohan and Benjamin, 1980a,b).

In order to investigate the hypothesis that food-induced arousal could be mediated by release of serotonin

from the CGCs of Lymnaea we adopted a different approach than that used previously for either Lymnaea or Aplysia . This involved reducing the levels of serotonin in the CGCs by injecting the substance 5,6-dihydroxytryptamine (5,6-DHT) into intact snails and assaying by behavioural tests the effects on food-induced arousal. This approach was successfully used previously in our similar experiments with Helix (Kemenes and S.-Rózsa, 1987; Vehovszky et al., 1988).

In parallel experiments the effects of the CGCs on feeding neurones (mainly motoneurones) were tested in isolated ganglia dissected from snails following drug injection, to see if any changes in behaviour could be directly attributed to the CGCs. The results showed that reduced levels of serotonin in the CGCs were correlated with a reduction in feeding arousal responses to sugar and that excitatory responses in feeding motoneurones were also lost at the same time. The preliminary electrophysiological data do not provide an explanation for the behavioural results but at least suggest that the CGCs rather than some other serotonin-containing neurones are involved in the behavioural response to drug injection.

MATERIALS AND METHODS

The tryptamine derivative 5,6-dihydroxytryptamine creatinine sulphate (SIGMA) was dissolved in HEPES-buffered saline (Benjamin and Winlow, 1981) containing 0.5 mg ml^{-1} ascorbic acid as an antioxidant. Fifty snails of 3.0 to 1.5 g body weight were injected with 25 to 50 µl of the drug solution. This is equivalent to 200 mg kg^{-1} body weight 5,6-DHT dose. A group of 50 control snails was injected with the carrier solution alone. Behavioural feeding tests were performed on both groups from 25 min up to 28 days after the injection. Feeding movements (opening, rasping and closing of mouth) of an individual snail were stimulated by

pipetting sucrose (10^{-3} mol l^{-1} final concentration) into a chamber and permanently recorded using the previously described methods (Kemenes et al., 1986). Snails that did not start feeding within 2 min after the sucrose stimulus were considered as "non-responding". Seven to 10 snails were tested in each experiment and the mean latency to the first bite ($\bar{L}$), mean duration of the first 20 bites ($\bar{D}_{1-20}$) and the mean of the first 20 interbite intervals ($\bar{I}_{1-20}$) were computed. One-tailed paired and unpaired t-tests (Minitab Statistics Package) were performed on the data to establish significance levels for differences between control and experimental groups. The feeding tests were followed by histochemical, biochemical and electrophysiological experiments carried out on individually labelled snails from the control and experimental behaviourally tested groups.

To measure monoamine fluorescence we used the histochemical glyoxylic acid procedure (Axelsson et al., 1973) modified by Audesirk (1985) on Lymnaea whole mount preparations. Changes in 5-HT fluorescence were monitored in the cerebro-buccal connectives and the latero- and ventrobuccal nerves from photographed material. Dopamine (DA) fibers also occur in the cerebro-buccal connectives and the buccal nerves and the occurrence of DA in these fibers was also monitored using suitable filters to allow their green fluorescence to be separated from the yellow 5-HT fluorescence.

DA, 5-HT and 5,6-DHT levels in control and experimental ganglia were monitored using previously described HPLC methods (Saller and Salama, 1984). The following pieces of tissue were analyzed: buccal ganglia, cerebro-buccal connectives together with the latero- and ventrobuccal nerves and the cerebral ganglia.

Electrophysiological experiments, similar to those described previously (McCrohan and Benjamin, 1980b) on isolated CNS preparations from control and 5,6-DHT injected

snails were used to study changes in the synaptic connections between CGCs and feeding motoneurones.

RESULTS

Changes in feeding behaviour after 5,6-DHT injection

Three hours after the injection the mean latency to the first bite after sucrose presentation ($\bar{L}$) and the mean of the first 20 interbite intervals ($\bar{I}_{1-20}$) were significantly longer in the 5,6-DHT injected than in either the pre-injection or same day's vehicle injected control group ($p < 0.01$, paired and unpaired t-test, respectively; Fig.1a,b). At the same time the mean duration of the first 20 individual bites ($\bar{D}_{1-20}$) was significantly shorter in the experimental than in the control snails ($p < 0.001$, paired and unpaired t-tests, respectively; Fig.1c). The percentage of non-responding snails (shaded bar in Fig.1a) was extremely high (64%) in the experimental group as compared with the vehicle control group (11%). The only other time when significant differences were observed in all four parameters between the experimental and control groups was 15-16 days after the injection. At this time, the $\bar{L}$ and $\bar{I}_{1-20}$ values were significantly greater than either in the preinjection control ($p < 0.01$ and $p < 0.001$, respectively), or vehicle-injected snails ($p < 0.001$; Fig.1a,b). The duration of individual bites again was significantly shorter in the experimental group than in either control group ($p < 0.001$). By the 21st day after the injection the number of non-responding animals, the $\bar{L}$ values and the $\bar{I}_{1-20}$ values were not significantly different in the vehicle- and 5,6-DHT injected groups (Fig.1a,b). The duration of the individual bites, however, remained significantly shorter in the experimental group ($p<0.01$), even 28 days after the 5,6-DHT injection (Fig.1c).

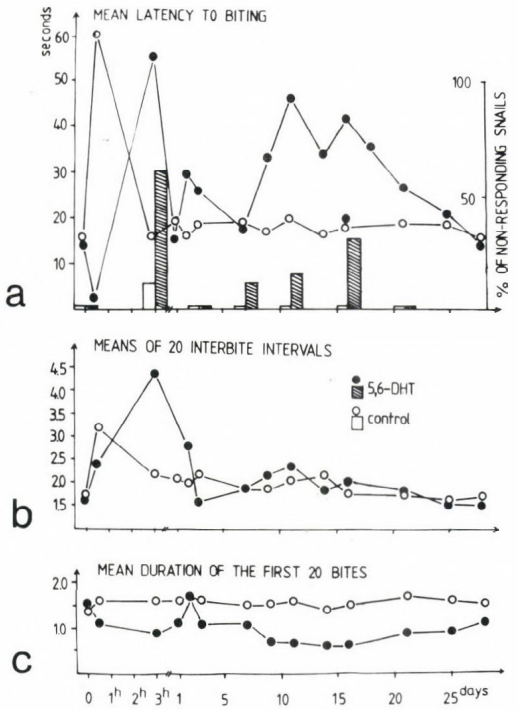

Fig.1 Changes in four different parameters of the feeding behaviour in control and 5,6-DHT injected snails at different times after the injection. Significant changes in all four parameters together could be observed 3 hours and 15-16 days after the injection (see text).

Changes in 5-HT and DA fluorescence in the cerebro-buccal connective and latero- and ventrobuccal nerves after 5,6-DHT treatment

No weakening of either 5-HT (CGC axonal processes) or DA fluorescence (fibres of unknown cells) was observed for up to 12 days after the 5,6-DHT injection (Fig.2a). Between the 14th and 16th day, however, 5-HT fibers could not be detected in the cerebro-buccal connective and latero- and

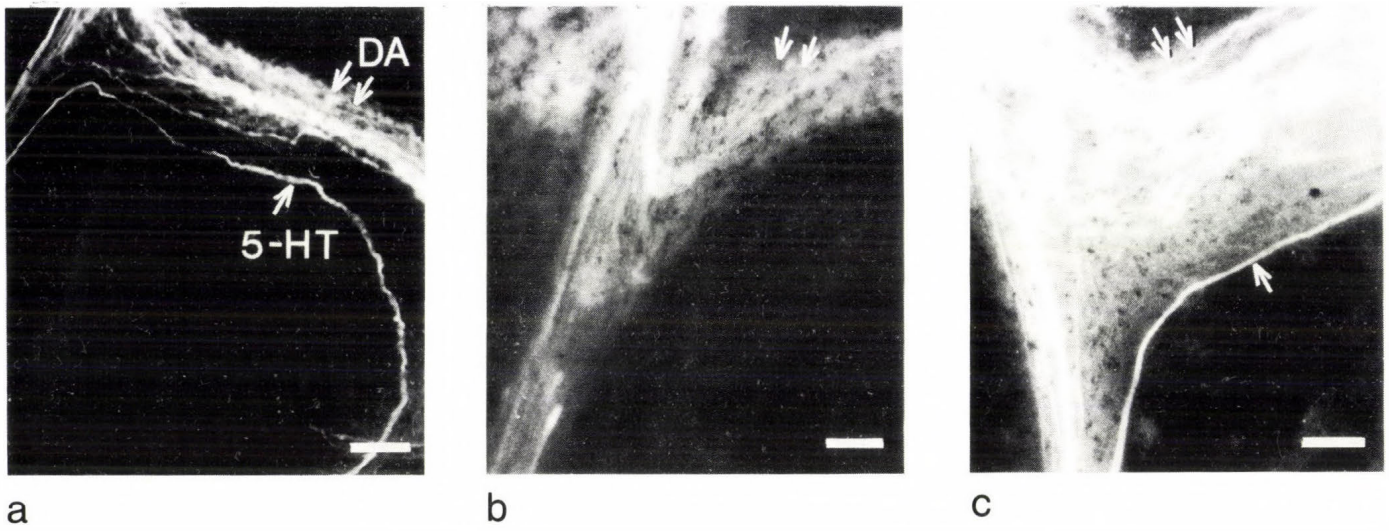

Fig.2 In glyoxylic acid treated preparations DA and 5-HT fluorescent fibers could be found together in the cerebro-buccal connective and latero- and ventrobuccal nerves in control snails and shortly after 5,6-DHT injection (a, DA: double white arrow, 5-HT: white arrow, identified on colour photographs of the same preparation). Fifteen days after the drug injection 5-HT fluorescent fibers could not be seen in the same structures (b). Twenty-two days after the drug injection 5-HT fluorescence could again be detected in the connective and nerves (c). The 5-HT fibers are axonal branches of the main CGC axon projecting to the buccal ganglia and buccal mass (McCrohan and Benjamin, 1980a). Calibration bars: a and b 50 µm, c 20 µm).

ventrobuccal nerves while DA fluorescence was still present in the same structures (Fig.2b). By the 18th day after the injection 5-HT fluorescent fibers of the CGCs could again be seen in the connective and nerves (Fig.2c). Throughout the whole period (1-18 days) both 5-HT and DA fluorescence was seen in controls.

Differences in 5-HT and DA levels measured by biochemical techniques

There was no difference between the 5-HT contents of buccal ganglia dissected from control and experimental snails 3 hours, 15 days and 22 days after the injection (Fig.3 , B.G.). In the cerebro-buccal connectives and latero- and ventrobuccal nerves, however, 3 hours after the injection, 5-HT levels were 1.63 ± 0.58 times higher ($p < 0.005$) in the experimental than in the control snails (Fig.3 , N.). By the 15th day this relationship had been reversed, in the experimental snails 5-HT levels were only 61 ± 14 percent of those measured in control snails ($p < 0.0005$). By the 22nd day the difference in 5-HT levels had disappeared. In the cerebral ganglia, 3 hours after the injection and parallel with the significant increase of 5HT level in the cerebrobuccal connective, a significant ($p < 0.005$) decrease was found in the 5-HT level in experimental snails (78 ± 6.7% of control, Fig.3 , CR.G.). Again, this decrease was transient with no difference between experimental and control groups in 5-HT levels 15 and 22 days after treatment.

The 5,6-DHT injection also had an effect on the DA levels in the above mentioned tissues (Fig.3). Three hours after the injection a transient but very strong (about 130%, $p < 0.0005$, Fig.3 , N.) increase was observed in the cerebrobuccal connectives and latero- and ventrobuccal nerves. This was accompanied by a significant ($p < 0.05$)

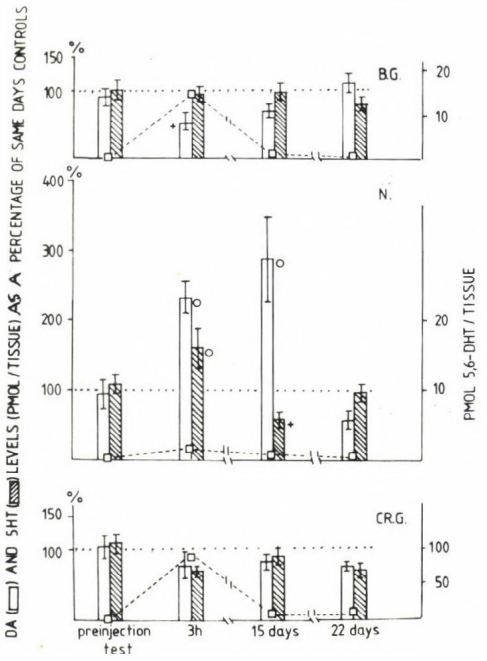

Fig.3 5-HT (shaded bar) and DA (empty bar) levels as a percentage of same days' controls in 5,6-DHT injected snails at different times after 5,6-DHT injection. 5,6-DHT levels were also measured in the experimental animals (empty squares, right scale). B.G.: buccal ganglia; N.: cerebrobuccal connective and latero- and ventro-buccal nerves; CR.G.: cerebral ganglia. o= transmitter level higher than in control (100%, dotted line), $p < 0.005$ to 0.0005; += transmitter level lower than in control, $p < 0.005$ to 0.0005.

decrease of DA levels in the buccal and cerebral ganglia of experimental snails (Fig.3, B.G. and CR.G.). The only other time when difference was found in DA levels between control and experimental snails was 15 days after the injection. At this time, however, the only tissue that had different DA

levels in control and experimental snails was the connective and the nerves (Fig.3 , N.). The DA level in these structures was about 3 times higher ($p < 0.0005$) in the experimental snails than in the control ones. This was also the time when 5-HT was significantly depleted from the connective and nerves.

The highest level of free 5,6-DHT in all nerve tissues was measured 3 hours after the injection of the drug (Fig.3) and thereafter it rapidly declined.

Changes in the synaptic connections between CGCs and buccal motoneurones in 5,6-DHT and vehicle-injected snails

The synaptic connections from the CGCs to various motoneurones described in detail by McCrohan and Benjamin (1980b) were found to be intact for up to 12-14 days after the treatment with either 5,6-DHT or vehicle (Fig.4a,b). However, 15 or 16 days after the injection with 5,6-DHT, the synaptic inputs from the CGCs to buccal motoneurones were transiently blocked (Fig.4c,d). Accompanying this blockade of synaptic transmissison between CGCs and motoneurones was a reduced level of spontaneous central pattern generator (CPG) activity recorded in the motoneurones (Fig.4e). Control snails dissected on the same days had normal CGC-buccal motoneurones, excitatory synaptic connections and patterns of CPG driven burst activity (not shown in Fig.4). In snails dissected 22 days after 5,6-DHT injection the synaptic connections between CGCs and buccal motoneurones were again as strong as in control animals (Fig.4f).

DISCUSSION

The most striking responses to 5,6-DHT occurred from 15 to 18 days after injection. Here specific reductions in behavioural responsiveness were correlated with a reduction in 5-HT content in CGC axons measured by histofluorescence

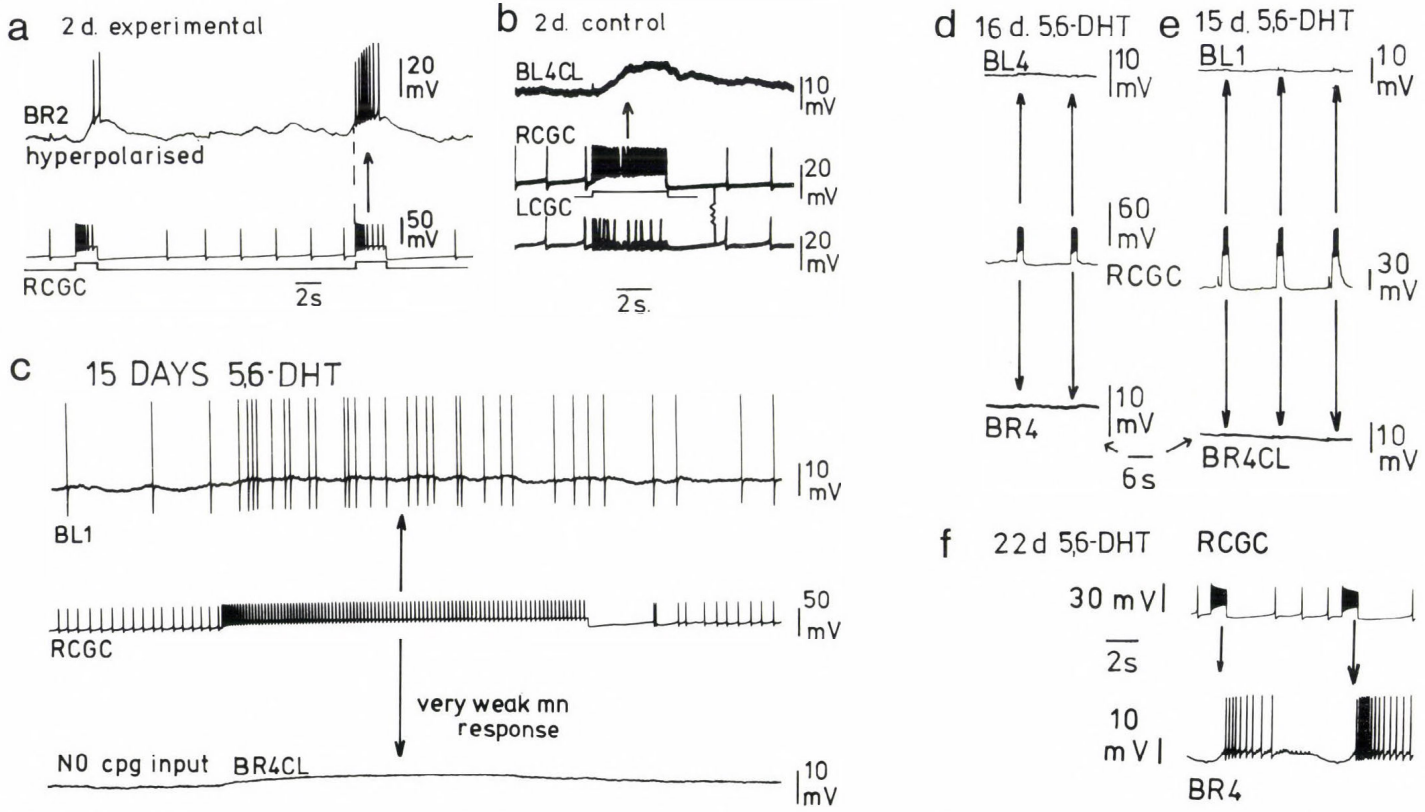

Fig.4 Shortly after 5,6-DHT injection synaptic connections between CGCs and buccal motoneurones in the isolated preparation are still intact (a,b). Fifteen and 16 days after drug injection these connections are transiently blocked (c,d,e) and no CPG input can be found on the motoneurones (c). The connections are normal again 22 days after 5,6-DHT injection (f).

and a drop in HPLC assayed levels of 5-HT in nerve trunks carrying the same fibres. At the same time (15-16 days) the synaptic responses of motoneurones to CGC activation were reduced or lost and the general excitability of the feeding CPG was reduced. A similar transient blockade between the cerebral serotonergic cells and buccal neurones has been described in Helisoma (Gadotti et al., 1986) and Helix (Vehovszky et al., 1988) following 5,7- or 5,6-DHT treatment. This suggests that the release of 5-HT from the CGCs plays some important role in determining the response of snails to food. 5,6-DHT injection increased the proportion of nonresponding snails, increased the latency to respond and reduced the duration of the bite (scrape) phase of the feeding cycle. These carefully measured changes in behaviour might be considered as a reduction in "arousal" of the animal to food and compared with a similar classification of behaviour in Aplysia (see Susswein et al., 1978) .

A similar set of behavioural changes occurred within 3 hours of 5,6-DHT injection but these cannot be explained by simple reduction in central serotonergic transmission of the type discussed above. This is because histofluorescence studies still showed 5-HT in CGC axons and transmission between CGCs and motoneurones was normal. Paradoxically, there was an actual increase in HPLC measured levels of 5-HT in the cerebro-buccal connectives and buccal nerves leading to the periphery although an overall decrease in 5-HT level did occur in cerebral and buccal ganglia. One possible explanation for these results from the HPLC assay is that 5,6-DHT was causing some increase in the peripheral release of 5-HT thus reducing the central concentration of the transmitter. However, it is not easy to see from this how the detailed short-term behavioural changes were brought about.

It is interesting that 5,6-DHT treatment in the 15-16 day animals only reduced 5-HT levels in the nerves and never

completely depleted cell body content. Serotonin depletion by toxic serotonin analogs appears to be most effective distally to the cell bodies (Gadotti et al., 1986). This may reflect the higher levels of 5-HT in the cell bodies but a more likely explanation is that 5,6-DHT affected mainly the release rather than the synthesis of 5-HT. In Lymnaea, unlike in Aplysia (Ono and McCaman, 1984), Helisoma (Murphy et al., 1985) and Helix (S.-Rózsa et al., 1986) the buccal ganglia also contain serotonergic cells that became characteristically pigmented (Glover and Kramer, 1982; Lent and Dickinson, 1984; S.-Rózsa et al., 1986) about three weeks after the 5,6-DHT injection (our unpublished observations). This can account for the failure to detect depleted 5-HT levels in the buccal ganglia of Lymnaea after 5,6-DHT treatment. It should not be forgotten that the 5,6-DHT treatment also influenced the levels of DA in the Lymnaea feeding system. However, the significance of this will not become clear until we know something about the role of this transmitter in the defined neurones of the feeding circuit.

SUMMARY

The drug 5,6-dihydroxytryptamine (5,6-DHT) is known to cause the selective ablation of serotonergic pathways in both vertebrates and invertebrates and we used this neurotoxin as a tool to study the role of serotonin-mediated modulatory mechanisms in the feeding behaviour of Lymnaea. We assayed the build-up of food-induced arousal in intact untreated, 5,6-DHT-injected (200 mg/kg) and vehicle-injected snails at different times after the injection. Parallel with the behavioural tests electrophysiological experiments were carried out on the serotonergic cerebral giant cells (CGCs) and various motoneurones and modulatory interneurones in the isolated CNS preparations. The behavioural and physiological data were correlated with the

results of the histochemical glyoxylic acid technique used for localization of monoamines such as serotonin (5-HT) and with the results of HPLC measurements of 5,6-DHT, 5-HT and dopamine levels in the ganglia of the CNS. The effect of the 5,6-DHT was time-dependent and largely reversible. Between the 15th and 18th days after the injection of the drug the number of non-feeding animals, the latency of the feeding response and the interbite intervals were significantly longer and the duration of bites shorter in the experimental than in the control groups. In the same interval a weakening of the glyoxylic acid-induced yellow 5-HT fluorescence in the more peripheral serotonergic pathways and a depleted 5-HT level in the same structures were observed. A recovery from this state started after the 18th day following the injection and at the same time a strong brown pigment appeared in the serotonergic cells in each ganglion. Associated electrophysiological experiments revealed preliminary evidence for a blockade of synapses between the serotonergic giant cells and motoneurones and a reduction of excitability in the feeding pattern generator. This followed the same time course as the behavioural and other effects.

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DISCUSSION

ELLIOT, C.J.H.: In the electrophysiological experiments, on day 14 the motoneurons responded to CGC stimulation, but not on day 15. How do you explain these results?

KEMENES, G.: These are preliminary results and only a few snails have been sampled after each behavioural test. However, the only time when we observed complete blockade of synaptic connections between CGCs and motoneurones was between the 15th and 18th day and never before or after this time. On the other hand, I pointed out in my talk that in 14-day electrophysiological experiments we already found an increased latency of the motoneurone responses after CGC stimulation.

BENJAMIN, P.R.: We were not trying to suggest that the synaptic response in motoneurones suddenly disappeared at one particular day but there was a gradual decrease in response after 5,6-DHT injection. This gradually recovered with time.

SYED, N.I.: Are there two SOs (slow oscillator cells) in the right and left buccal ganglion of Lymnaea, respectively, as you showed in your slide?

KEMENES, G.: No, there is only one SO in either the left or the right buccal ganglion. This is an old slide made at the time when it was thought that SOs are a symmetrical pair of neurones in the buccal ganglia.

LUKOWIAK, K.: Daniela Gadotti in a published paper showed results similar to what you presented here. The only difference being that 5-HT levels in the buccal ganglia of Helisoma were decreased by 95% after 5,7-DHT injection. Why do you think your results are different?

KEMENES, G.: This is because unlike in Helisoma, there are 5-HT containing neurones in the Lymnaea buccal ganglia and 5-HT

depletion by 5,6- or 5,7-DHT appears to be most effective distally to the cell bodies.

LUKOWIAK, K.: In addition to 5-HT there appear to be many other neural-active agents which regulate "fictive feeding" on patterned motor activity in Helisoma. Thus it is sometimes surprising to see that by removing just one of these agents feeding would cease. In Helisoma we noted that although 5,7-DHT did somewhat alter feeding, the animals did continue to eat.

KEMENES, G.: It is very likely that in Lymnaea too, mediators other than 5-HT play an important role in the regulation of feeding. Removing the 5-HT from the system resulted in reduced responsiveness and slower feeding in most snails and only about 30% of the animals did not respond to food within 2 minutes after its presentation.

BALABAN, P.: It is known that in vertebrates 5,6-DHT is more toxic than 5,7-DHT. Why did you use 5,6-DHT?

KEMENES, G.: 5,6-DHT is slightly more toxic in rats than 5,7-DHT but no one has ever proved the same in snails. In our careful preliminary tests we found no aspecific toxic effects of the 5,6-DHT dose applied in our experiments and definitely no difference was observed between the behavioural effects of 5,6- and 5,7-DHT. On the other hand, 5,6-DHT induces a darker pigment in the 5-HT containing neuronal bodies and this helped us more to identify serotonergic elements in the system.

WINLOW, W.: Did 5,6-DHT have any effect on locomotion in Lymnaea?

KEMENES, G.: Yes. Shortly after injection the snails became hyperactive and a characteristic, rather uncoordinated "wriggling" was observed for about 20 minutes. In the later phase of the treatment, however, no abnormal forms of locomotion were seen.

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MODULATION OF MEMBRANE PLASTICITY BY LOW MOLECULAR WEIGHT NEUROTRANSMITTERS AND OPIATE PEPTIDES IN THE HELIX NEURONS

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Lately, co-existence of more than one neurotransmitter in a single neuron has become the rule rather than the exception. The neurotransmitters, co-existing within the same or releasing from different neurons, can interact both at pre- or post-synaptic sites in a functionally coordinated manner. The co-existence of several transmitters in a single neuron led to the recognition that the transmission of messages across the synapses is a more complicated event than was previously presumed. The functional significance of multiple putative messengers is not very well understood, neither is the question whether these messengers can produce selective and differential responses. Most frequently the peptides alone have no apparent effect but modulate the classical physiological processes regulated by the neurotransmitters. Nevertheless, there are some indications that the peptides could be involved in filtering the information at the synapses.

Using the semi-intact preparation of Helix pomatia it was shown that in the spring, during the reproductive phase of the animals, some neurons display a high-frequency burst of firing which can cause a dysfunction of the pathways operating with monoamines or acetylcholine as a neurotransmitter (S.-Rózsa, 1982). Peptides were found to elicit a similar burst of firing and modulation in the effectiveness of low molecular weight neurotransmitters (S.-Rózsa, 1982; Salánki and S.-Rózsa, 1986).

During studies on the site and mode of interaction of the peptides and classical neurotransmitters in the CNS of Helix pomatia two groups of neurons were identified which differed

in their plasticity (S.-Rózsa and Dyakonova, 1987). Some of these neurons are also involved in the regulation of visceral functions (S.-Rózsa and Zhuravlev, 1981). The aim of the present investigations was to study the interaction of various peptides and neurotransmitters in regard to the modulation of the plasticity of identified habituating neurons.

MATERIALS AND METHODS

The experiments were carried out on the identified habituating and non-habituating cells of Helix pomatia L. The habituation of the neurons was tested using intracellular stimuli with depolarizing current pulses of threshold value (duration 1 s, frequency 0.2 Hz). The current which generated a single action potential on its first trial was defined for threshold. The neurons were penetrated with two microelectrodes filled with 2.5 M KCl having a resistance of 10-20 MOhm. The conventional microelectrophysiological set up was used to record and display intracellular electrical activity of the neurons.

The experiments were performed on the non-isolated and entirely isolated identified neurons. The identification and isolation of the cells have been described elsewhere (S.-Rózsa and Dyakonova, 1987). In some experiments the effect and interaction of the drugs were compared on the isolated and non-isolated habituating neurons in the same preparation to separate their synaptic and somatic actions. The semi-intact preparation was also used including CNS, cardio-renal and respiratory systems with their connecting nerves (S.-Rózsa and Zhuravlev, 1981).

RESULTS

Distribution of habituating and non-habituating neurons in parietal ganglia of Helix

The two groups of neurons differing in their plasticity are located on the dorsal surface of the two parietal ganglia

(S.-Rózsa and Dyakonova, 1987). The non-habituating cells are concentrated on the upper part of the left parietal ganglion, while the non-habituating neurons are scattered along the midline of the left and right parietal ganglia (Fig. 1).

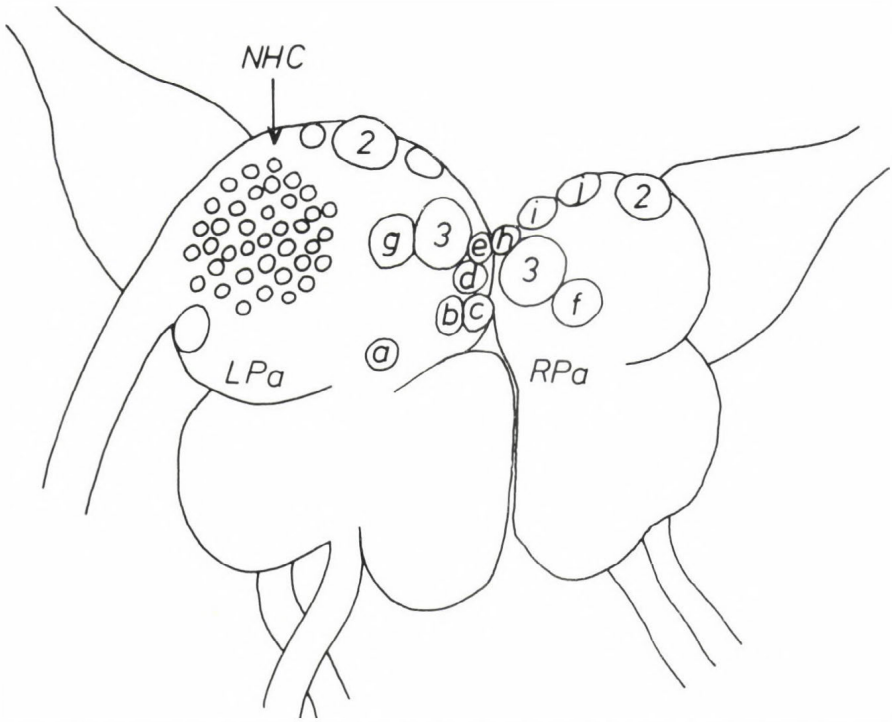

Fig. 1. Distribution of the habituating (cells 2 and 3, a-j) and non-habituating (NHC) neurons on the dorsal surface of the left (LPa) and right (RPa) parietal ganglia of Helix pomatia.

The non-habituating cells have a light pigmentation. Following insertion of microelectrodes they can fire for several minutes, then become silent. The threshold current of non-habituating cells is lower than that of the habituating ones and corresponds to 0.1-0.01 μA. The response of non-habituating cells remains constant for several hours.

The habituating cells are presumedly neurosecretory as they have an opalescent white colour which is more intensive in spring. The white cells are usually silent but following inser-

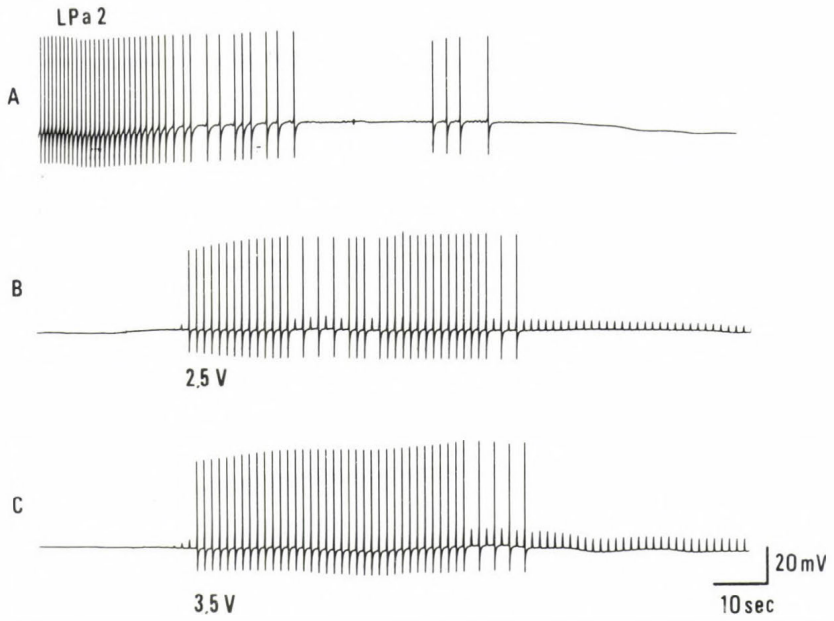

Fig. 2. Habituation to the intracellular stimuli at the neuron LPa2. A - following insertion with microelectrode the cell fires temporarily then becomes silent. B - habituation of the cell to threshold stimuli. C - habituation of the cell to elevated value of the stimuli.

tion of microelectrodes they could fire temporarily generating burst-like or tonic patterns (Fig. 2). As a rule, the burst pattern is preceded by membrane oscillations. Threshold current for their excitation was in the range of several nA. White cells are habituated to the intracellular stimuli within one to several minutes (Fig. 2).

Effect of neurotransmitters and peptides on the plasticity of habituating cells

In the presence of FMRFamide or serotonin the habituation of white cells was entirely eliminated. Here the inhibitory effect of FMRFamide is demonstrated on the habituation. As can be seen in Fig. 3 in the control, the neuron h habituated within one to several minutes of stimulation but following FMRFamide treatment (10^{-6} M) no habituation can be observed for over an hour

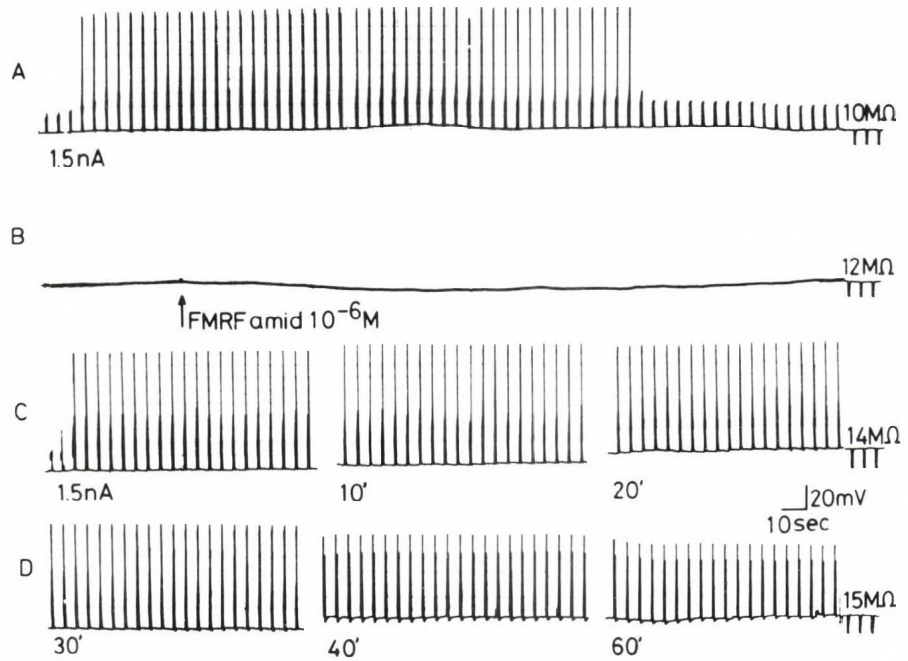

Fig. 3. Elimination of the habituation to intracellular stimuli with FMRFamide (10^{-6} M) treatment on the cell h. A - habituation of the cell to threshold stimuli. B - adding of FMRFamide to the bath caused slight hyperpolarization then slowly rising depolarization. C and D - in the presence of FMRFamide the cell failed to habituate to the intracellular stimuli for more than 1 hour. Resistance of the cell is shown in each record which was measured using hyperpolarizing current of 1.5 nA.

to the same stimuli. Simultaneously with elimination of the habituation the FMRFamide also caused gradual depolarization of the cell and increased its membrane resistance (Fig. 3). Both depolarization and elimination of the habituation evoked by FMRFamide were reversible.

Serotonin also eliminated habituation similarly to FMRFamide. The elimination of the habituation was linked in these cases to the alteration of the Ca-dependent K^+-conductance (S.-Rózsa and Dyakonova, 1987) using cyclic 3',5'-AMP as a second messenger.

Both serotonin and FMRFamide exert their effect on habituation without desensitization although in the elimination of habituation they show some differences (S.-Rózsa and Dyakonova,

1987) as FMRFamide may also change the Na+ and Ca^{2+}-conductances.

Not all neurotransmitters eliminate habituation on the white cells, on the contrary some substances can intensify habituation. As can be seen in Fig. 4, dopamine hyperpolarized the cell and an even more than 5-fold increase in the strength of the applied intracellular stimuli became ineffective in the presence of dopamine (Fig. 4).

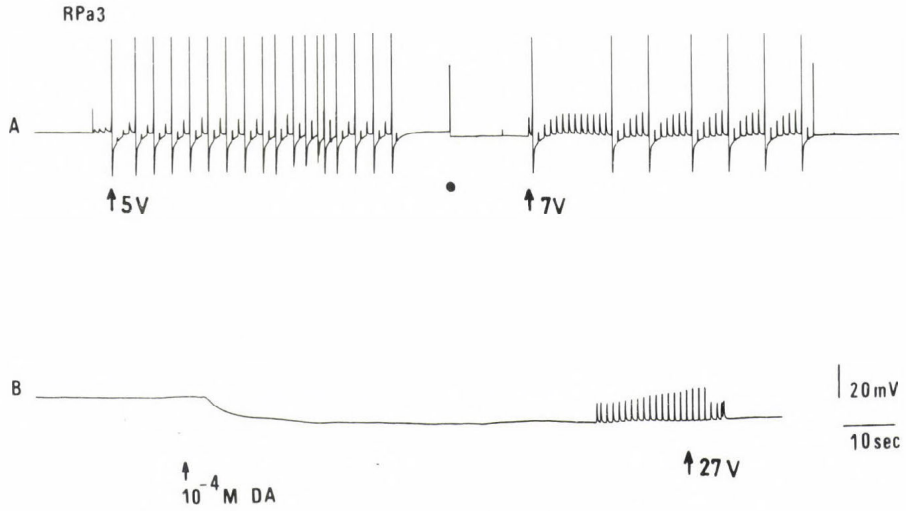

Fig. 4. Effect of dopamine on the habituation of the cell RPa3. A - habituation of the cell to threshold stimuli, then its effect to the increased stimuli. B - effect of dopamine (10^{-4} M) on the habituation. ● - 5 min intermission. Arrow - application of dopamine or stimulation of the cell.

The same effect was found on the habituation following morphine treatment (Fig. 5) when the strength of the stimuli was increased, but the habituation still remained. GABA was found to facilitate habituation in contrast to elevating the strength of the stimuli. However, the vasoactive polypeptide (VIP) was completely inactive in regard to the habituation as it failed to inhibit or enhance it (unpublished results).

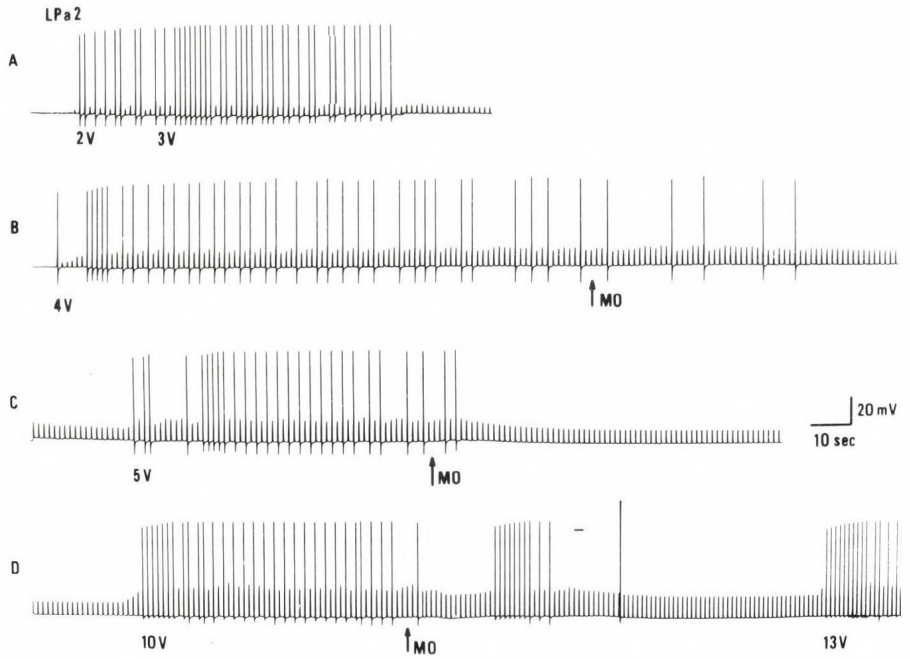

Fig. 5. Effect of morphine treatment on the habituation of the cell LPa2. Morphine treatment led to the increase of the threshold stimuli causing an effect on the cell, but habituation still occurred.

Site of action of substances eliminating the habituation of cells

In this series of experiments the neurons a, b and c were used which have common membrane properties and receive symmetric synaptic inputs (S.-Rózsa and Dyakonova, 1987). One of the cells was entirely isolated while the second one maintained its synaptic connection intact during the experiment on the same preparation, allowing the separation of the synaptic or somatic effects of the drugs influencing habituation.

The cells a, b and c have a membrane potential (MP) within the range of -30 to -70 mV, their firing pattern may show regular rhythm with varying frequency or may be burst-like coinciding with the oscillations of MP, or they may even be silent. The simultaneous registration of the activity of any two of the

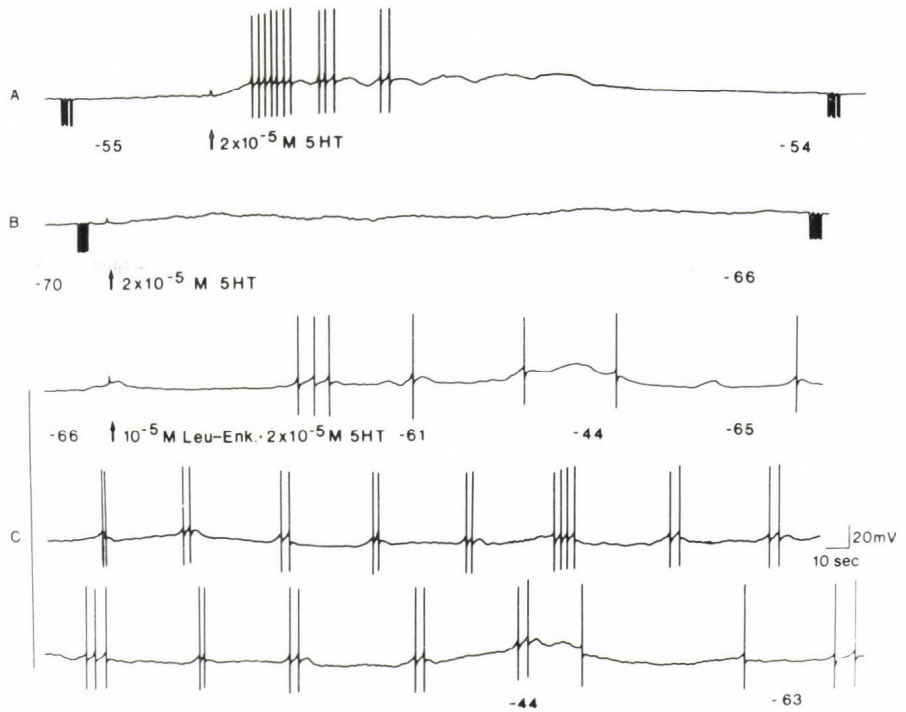

Fig. 6. Effect of serotonin on the cell c with intact synaptic connections. A - effect of serotonin ($2x10^{-5}$ M) at low MP value. B - application of serotonin at high MP value. C - effect of serotonin at high MP value in the presence of leu-enkephalin. Following leu-enkephalin treatment the serotonin effect appeared at high MP level, too. Recording C is a continuous one.

three neurons revealed the synchronous appearance of slow MP oscillations without electronic connections between them. The postsynaptic potentials and firing patterns were also found to be identical in the three cells in the same preparation.

At the moment of isolation, high frequency pacemaker firing appears in the cells a, b and c which continues for hours. The MP and membrane resistance of isolated cells remained near that measured in vivo.

The effect of serotonin on isolated and non-isolated cells differs considerably. In non-isolated cells serotonin (10^{-5}-10^{-4} M) was shown to generate MP oscillation and burst pattern in a voltage-dependent manner (Fig. 6). At MP more negative

than -55 mV serotonin caused only a moderate depolarization and decrease in membrane resistance. On the entirely isolated cell serotonin caused a large hyperpolarization of long duration, inhibition of the pacemaker activity and a significant decrease in membrane resistance (Fig. 7).

The somatic effect of serotonin was found to be mediated by cyclic 3',5'-AMP and this was demonstrated both on isolated and non-isolated cells (S.-Rózsa and Dyakonova, 1987).

On the non-isolated cell leu-enkephalin had an insignificant effect, while on the isolated cell leu-enkephalin caused depolarization as well as an increase in firing frequency and membrane resistance (Fig. 8).

Interaction of serotonin and leu-enkephalin on the habituating neurons

As described above on the non-isolated cell serotonin induces voltage-dependent MP oscillation and burst firing which cannot be observed at a MP value more negative than -55 mV. However, in cases where the ganglia are pretreated with leu-enkephalin, serotonin also elicits typical MP oscillations and burst pattern at more negative MP values (Fig. 6).

An opposite interaction can also be demonstrated when leu-enkephalin alone does not evoke MP oscillations, but following pretreatment with serotonin in a subthreshold concentration or after its washing out, leu-enkephalin can reproduce the serotonin effect (Fig. 8).

On the isolated cell the serotonin effect can also be modulated by leu-enkephalin. Serotonin produces long-lasting hyperpolarization stopping the spike activity during its presence in the bath. However, pretreatment of the ganglia with leu-enkephalin can reduce the serotonin effect to such an extent that only a short-term oscillation and temporary block of spike firing can be observed (Fig. 7).

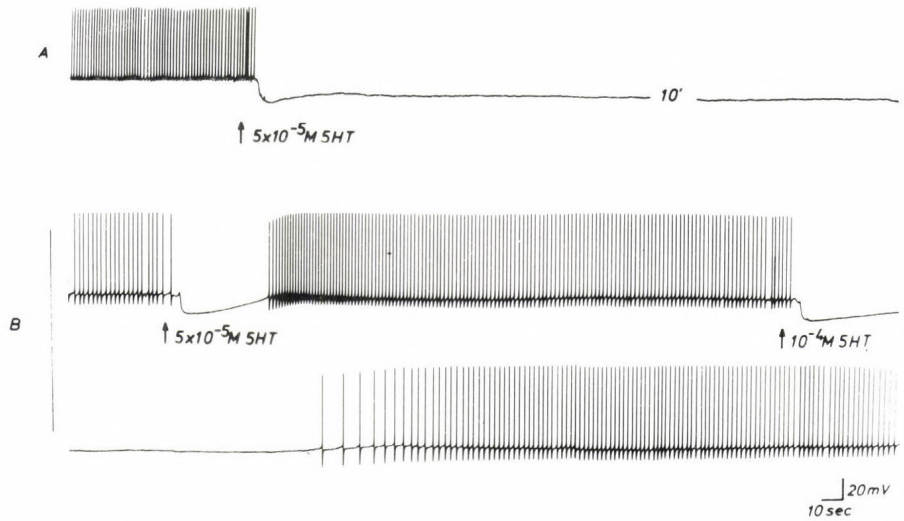

Fig. 7. Effect of serotonin on the isolated cell a. A - pacemaker activity of the isolated cell, then (↑) addition of serotonin causes a long-lasting hyperpolarization. B - in the presence of leu-enkephalin serotonin failed to evoke a long-lasting hyperpolarization and the pacemaker firing was restored within several seconds. In row B the two samples are the result of continuous recording.

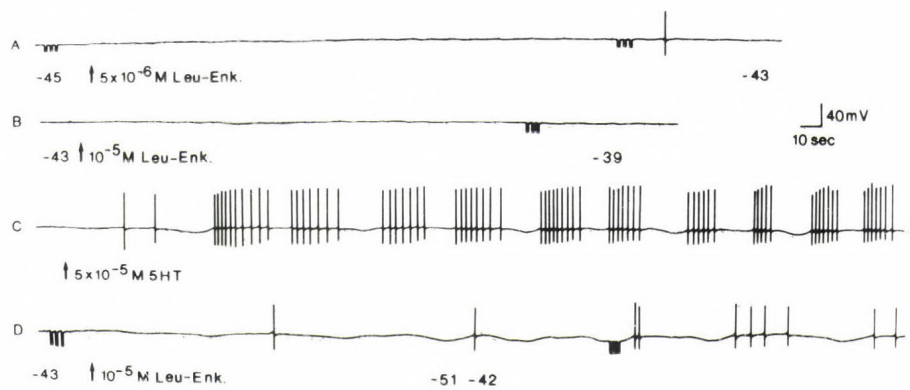

Fig. 8. Modulation of the effect of leu-enkephalin following 5HT treatment on the cell a. A and B - without serotonin only weak depolarization and slight increase in membrane resistance are caused by leu-enkephalin. C - serotonin generates oscillatory waves and burst firing on the same cell. D - following the washing out of serotonin, leu-enkephalin can reproduce the serotonin-like effect.

Relationship between firing pattern of the habituating cells and the regulation of pneumostoma

As the habituating cells include some of the neurons regulating the movements of the pneumostoma (S.-Rózsa and Zhuravlev, 1981), it was investigated whether the oscillatory waves of the cells a, b and c can also be connected to the regulation of the pneumostoma.

Our experiments proved that the MP oscillations of these cells either appearing spontaneously or elicited show a cor-

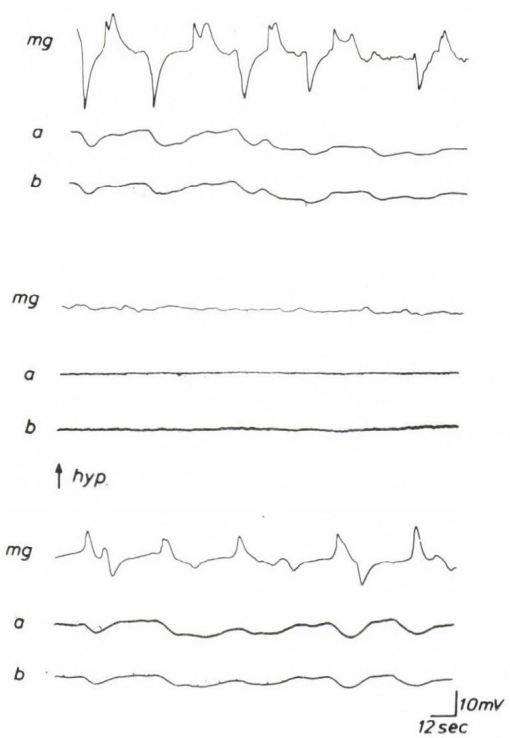

Fig. 9. On semi-intact preparations the synchronous oscillations of the MP on cells a and b are in close correlation with the myogram (mg) reflecting the opening and closing of the pneumostoma (above). Upon long-lasting hyperpolarization of the cells, the components of the myogram connected to the opening and closing of the pneumostoma are missing and only small local contractions are present (centre). After the hyperpolarization is abolished the opening-closing rhythm of the pneumostoma and MP oscillations were restored (below).

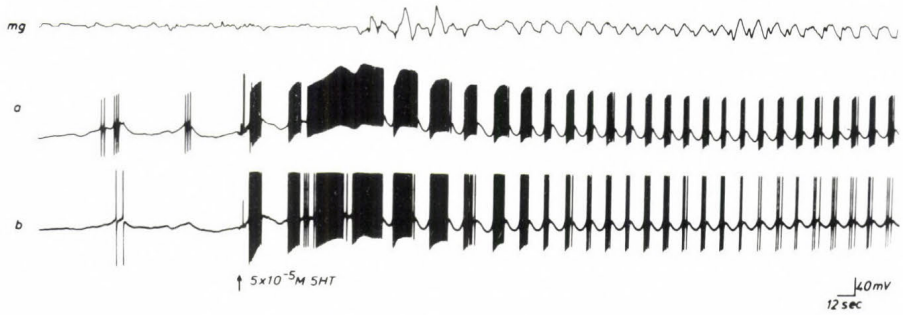

Fig. 10. In semi-intact preparations with cells a and b following application of serotonin synchronous MP oscillations and burst firing can be observed coinciding with the synchronous opening-closing rhythm of the pneumostoma.

relation with the movements of the pneumostoma. In Fig. 9 it can be seen that the myogram reflecting the opening-closing rhythm of the pneumostoma can be recorded synchronously with the oscillatory waves generated in cells a and b. Here the rising phase of the oscillatory waves correlates with the opening of the pneumostoma, while the decay corresponds to its closing (Fig. 9). The opening-closing rhythm can be recorded as long as the MP oscillation is maintained in cells a and b (Fig. 9). However, during long-lasting hyperpolarization of the cells, the MP oscillation disappears and simultaneously the rhythm of the pneumostoma ceases and only some local contractions remain (Fig. 9).

On the cells a and b the serotonin-evoked MP oscillations also correlate with the movements of the pneumostoma (Fig. 10). The spontaneous generation of the MP oscillation can be a result of an activation of the unidentified inputs of cells a, b and c regulating the respiratory rhythm.

DISCUSSION

The results showed that certain neurotransmitters and peptides can alter the plasticity of the habituating neurons in the CNS of Helix pomatia L. Among them serotonin and FMRFamide

were found to eliminate, while dopamine and morphine to intensify the habituation on the identified white cells. At the same time, the opiate peptides leu- and met-enkephalins are involved in the modulation of the above effects or in the long-lasting changes sharing an intracellular site of action.

The data obtained show that the habituating cells can be involved in the regulation of complex integrative processes connected with rhythmic alterations controlled by a large number of neurons. The great variety of the functional state, complex pattern of firing, intense and varying synaptic activity and MP oscillations of these cells support the above suggestion. The habituating cells were found to be involved mainly in the regulation of the visceral function (S.-Rózsa and Zhuravlev, 1981).

Among the agents studied FMRFamide and serotonin were able to cause oscillatory waves on the habituating cells with intact synaptic connections, but on the isolated cell neither serotonin nor FMRFamide had such an effect. Leu- or met-enkephalin failed to elicit MP oscillation themselves, but shifted the MP value to the level required for the 5HT effect and so contributed to the appearance of its action; e.g. altered its effectiveness. This means that enkephalins depending on the initial MP level may facilitate the generation of the 5HT-evoked MP oscillation and burst firing, or conversely they can eliminate both if the MP falls below a critical value. This kind of modulation can be a result of changing the electrical properties of the soma membrane. The long-lasting but persistent depolarization and increase in membrane resistance caused by enkephalins can serve as a mechanism to change the system to the new functional level and to maintain it there for a long period.

Our results are consistent with the suggestion that only the substance causing membrane oscillations and burst firing are able to eliminate the habituation of the cells to intracellular stimuli. This kind of peptide effect is rather common and has been demonstrated for both invertebrate and vertebrate neurons (Gainer, 1977; Strumwasser, 1982; Bunney, 1987; etc.). This membrane oscillation can contribute to the "closing" of some pathways in the course of normal nerve functioning as it can

determine whether the cell will respond or not to the low molecular weight neurotransmitters. This mode of action can contribute to the mosaic-like functioning of the units in the neural network (S.-Rózsa, 1982; 1987).

In the case of the regulation of visceral function this kind of switching of the system from one mode of activity to another (answer - no answer) has a primary importance, as various units can have a "rest" or "work" state and in this way some of the units will be ready for regulation at any time. The pneumostoma movement is only one of the examples of such regulation, where the habituation of the cell can serve as a basis for stopping the regulation (giving a rest for the regulated organ), and the neurotransmitters (5HT or peptides) causing membrane oscillations can lead to the termination of inactivation in the regulation (see Figs 9 and 10). In the nervous system both the "switch off" and "turn on" of certain units can be regulated by neurohumoral agents, where the peptides can be involved both through regulation of the MP level and mode of firing the cells.

Earlier, while measuring the liberation of more than one substance into the heart during stimulation of the extracardiac (intestinal) nerve in Helix pomatia, a "stepwise" mode of action of neurotransmitters was suggested (S.-Rózsa, 1969), including the interaction between various classes of biologically active agents. It is now more and more justified that also in the central nervous system a "mixture" of regulatory substances is involved in any kind of excitation or inhibition. Simple preparations such as the habituating cells with known regulatory function may hopefully contribute to the elucidation of the role of these "mixtures" of transmitters.

SUMMARY

Based on their habituation to intracellular stimulation, two cell groups were identified in the left and right parietal ganglia of Helix pomatia L. (Mollusca, Gastropoda). One of them showed fast habituation to the intracellular stimuli, while the

other did not habituate. The habituating cells belong to the neurosecretory type.

Serotonin and FMRFamide were found to eliminate the habituation, while dopamine, GABA and morphine intensified the habituation on the same neurons. Some other peptides (VIP, β-receptor peptide) were ineffective on habituation.

The substances were found to eliminate habituation on the white cells and were able to cause membrane oscillations and burst firing on the same cells. The membrane oscillations evoked spontaneously or by neurotransmitters appeared only at low membrane potential level (between -35 and -55 mV). Membrane oscillation has never been observed on the entirely isolated habituating cells.

The effect of leu- and met-enkephalin was expressed to a greater extent on the entirely isolated cells and had an intracellular target.

In the habituating cells with intact synaptic connections enkephalins being nearly ineffective themselves were able to reproduce the earlier serotonin effect.

On the isolated habituating neurons 5HT caused a long-lasting inhibition of the pacemaker activity antagonized by enkephalins. Dibutyryl 3',5'-AMP and isobutylmethylxanthine were able to mimic the leu-enkephalin effect.

On the habituating cells a, b and c the membrane oscillation and burst firing were found to be connected to the regulation of the movements of the pneumostoma. The habituation on these cells can contribute to the mosaic-like activation of the units in the neural network regulating visceral organs, assuring in this way permanent functioning of the regulatory system.

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DISCUSSION

ALKON, D.L.: 1. Would it not be useful to distinguish between "plasticity" or modification and modulation? The former would refer to neuronal and/or behavioural changes which significantly outlast the conditions by which they were induced. By contrast, modulation, which you seem to have described, persists only (or largely) during the inducing condition (such as application of a hormone).

2. Would it not be helpful to determine which neutransmitters and/or neurohormones are actually released in situ under

the physiologic conditions of interest as compared to measuring effects of exogenous agents not necessarily involved during such conditions?

S.-RÓZSA, K.: 1. The term used in this case can be regarded as arbitrary. It is still a question of debate whether the plasticity, modulation and modification can really cover physiological events different in nature, or whether they form artificial categories created by scientists for simplification and a clearer understanding of the nerve function. In our system the cells showing habituation to intracellular stimuli are connected to the regulation of visceral function (e.g. respiration). This habituation can be eliminated by certain substances (serotonin, FMRFamide), while others (enkephalins) can increase or decrease their effect. Independent of the terminology it seems justified that this model can give a possibility to study the events involved in this alteration of the plasticity of the nerve membrane. However, as the changes caused by the agents eliminating habituation can outlast the application of the substances (oscillatory waves and burst firing), this supports the idea that this model can be useful in studying cellular mechanisms of elementary forms of learning.

2. Undoubtedly it would be helpful to determine the release of neurotransmitters or hormones liberated from or at the synapses of habituating cells, but at present it is impossible technically. Here the cell content has less importance than the substances located to the synapses terminating on the habituating cells. The identity of the endogenous agents as well as the substances used in our experiments cannot be stated at the moment but the interaction described between low molecular weight neurotransmitters and peptides may offer a model which can function in an intact nervous system as well.

TAUC, L.: What could be called an isolated neuron? What guarantees that the isolated cell used in your experiments has no synaptic connections?

S.-RÓZSA, K.: In this model system the only basis for separation of synaptic and somatic effects of the drugs was the comparison of the responses of the entirely isolated and non-isolated homologous cells in the same preparation. As the responses of the isolated and non-isolated cells were very different (see the serotonin effect) it can be stated that on the isolated cells the somatic effect is registered, otherwise the two effects should be the same.

WINLOW, W.: In answer to Prof. Tauc's comment about the localization of serotonin receptors on the axon hillock: we often find that isolation of neurons by this short length of axon is retracted and a perfectly spherical cell is formed. Presumably the serotonin receptors of the axon hillock are to be found on the surface of the "soma" in a case such as this.

van der WILT, G.: Can you relate the change of pneumostoma movements and the concomitant bursting activity in certain central neurons to the occurrence of a sensory input?

S.-RÓZSA, K.: In the experiments carried out in the semi-intact preparation of Helix pomatia it was observed that the membrane oscillation and burst firing of some habituating cells had close correlations with the movements of the pneumostoma. This membrane oscillation and burst firing had no endogenous origin and has never been observed in entirely isolated cells, but could be the result of activation of the inputs terminated on these cells. During hyperpolarization of the habituating cells the membrane oscillations and bursting stopped and the pneumostoma movements ceased, indicating their causal relationship. It was assumed that habituation of these cells is a mechanism for assuring permanent mosaic-like functioning of the neural network regulating visceral functions.

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MODULATION OF NERVOUS ACTIVITY IN LYMNAEA BY GENERAL ANAESTHETICS

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INTRODUCTION: THE NEED FOR A "MODEL" SYSTEM

Despite their clinical importance, the cellular and intracellular actions of general anaesthetics are poorly understood. Previous studies have utilised a plethora of different preparations depending on whether the study was carried out at the whole animal or cellular levels. Furthermore, a host of different anaesthetic agents have been applied to preparations often at doses unknown, uncontrolled or grossly in excess of those required to anaesthetize the intact animal (Trudell, 1985). In an attempt to gain fresh insights into the mode of action of general anaesthetics, we have used a single "model" invertebrate preparation, the pond snail **Lymnaea stagnalis** (L.), whose morphology facilitates studies of anaesthetic mechanisms at both behavioural and cellular levels (Winlow, 1984). Here we report the actions of general anaesthetics on aspects of the behaviour of **Lymnaea**, their effects on central neurones and on synaptic interactions between neurones.

CRITERIA FOR ANAESTHESIA IN LYMNAEA

Before proceeding with experiments on the isolated central nervous system, we determined the concentrations of halothane, enflurane and isoflurane which produced responses in the whole animal which could be equated to the clinically defined state of "anaesthesia". Most obvious and most important was that, in the presence of anaesthetic, **Lymnaea** loses the whole animal withdrawal response to gentle mechanical stimulation. Other criteria of anaesthesia observed, and upon which

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testing was based, were the abolition of the tentacular withdrawal response, the cessation of locomotion and shell movements associated with locomotion, the cessation of feeding movements and the overall relaxed manner of the animal. In general, all these criteria were expected to be fulfilled in the anaesthetized animal and recovery to be complete soon (i.e. certainly within 60 minutes) after withdrawal of the anaesthetic. No lingering after-effects or side-effects from exposure to anaesthetics were observed.

ED_{50} values for the whole animal withdrawal response were determined according to the methods of Cruickshank et al. (1985 and in preparation) and log dose response curves were constructed for each anaesthetic using 400 animals in each case. The ED_{50} values for halothane, enflurane and isoflurane at $20^{o}C$ are 0.83% v.v., 1.01% v.v. and 1.09% v.v. respectively (Girdlestone, 1986). These values fall well within the normal clinical ranges for each anaesthetic (de Jong & Eger, 1975) and indicate that **Lymnaea** is a suitable model preparation for the study of anaesthesia.

EFFECTS OF HALOTHANE ON THE FEEDING AND LOCOMOTOR SYSTEMS OF LYMNAEA

During the induction phase of anaesthesia, mammals pass through a brief excitatory phase when reflexes are exaggerated and respiratory movements increase and become irregular (Bowman and Rand, 1981). In the early stages of anaesthesia, or at low concentrations of halothane, specimens of **Lymnaea** passed through a phase of increased locomotory activity, during which the number of biting and mouthing movements also increased significantly (McCrohan et al., 1987). The activity is thought to be equivalent to the excitatory phase of anaesthesia described above.

GROSS EFFECTS OF HALOTHANE ON SPONTANEOUS NEURONAL DISCHARGES

At doses similar to those which anaesthetize the whole animal, halothane produced dramatic concentration-dependent effects which can be generalised for all cell types. Similar effects have also been observed with enflurane and isoflurane.

At low concentrations of halothane (0.25 - 0.75%), an interesting excitatory phenomenon was revealed (which may be the cellular equivalent

of the excitatory phase of anaesthesia mentioned above) rather than the expected classical depression of neuronal activity by anaesthetics. There was patterning of the spontaneous discharge of cells and, more often than not, characteristic random bursting of relatively high frequency (5 - 10 Hz), or rhythmic oscillations. In general, there was an excitatory response of all cell types (n=11) to low concentrations of halothane. This increase in excitability was maintained at low concentrations and maintaining the preparation in these concentrations for longer periods than the 5 minute perfusion period did not cause complete quiescence of cells.

Higher concentrations of halothane (i.e. those causing loss of the withdrawal response in 68-100% of animals), 0.75 - 2.00%, caused a generally depressant effect on the spontaneous activity of all the neurones examined (n=13). The spontaneous activity gradually declined to eventual quiescence, although in some cases this was accompanied by an initial, brief, excitatory period at the start of perfusion. Throughout this decline in the frequency of firing of action potentials, however, action potential amplitude was maintained at its normal value, as was membrane potential. Conversely, the after-hyperpolarization amplitude was reduced. The brief excitatory period was regularly seen again during the rinsing of the preparation with normal saline.

ANAESTHETICS MODIFY ACTION POTENTIAL TRAJECTORY

Halothane and the unorthodox, but nevertheless potent anaesthetic, menthol (Haydon & Winlow, 1981; Haydon et al., 1982), caused similar modifications of action potential shape. The pseudoplateau during repolarization was abolished together with the after hyperpolarization. In the case of evoked action potentials, the post-burst hyperpolarization (PBH) was also abolished. Both anaesthetics also decreased the frequency and sometimes the amplitude of evoked action potentials.

ANAESTHETICS INCREASE MEMBRANE CONDUCTANCE

Both menthol (Haydon et al., 1982) and halothane (Girdlestone, 1986) caused an overall increase in conductance determined by passing short, hyperpolarizing voltage pulses across the membrane. These effects were concentration dependent.

EFFECTS OF HALOTHANE ON SYNAPTIC TRANSMISSION

The cerebral giant cells (CGCs) of **Lymnaea** are strongly electrotonically coupled to one another and have chemical synapses on to many of the buccal motoneurones (McCrohan & Benjamin, 1980). The electrotonic coupling was unaffected even at very high halothane concentrations (8%). Equally the strong electrical synapse between VD1 and RPD2 (Benjamin & Winlow, 1982) remained unblocked in the presence of menthol (Haydon et al., 1982). Chemical synapses between the identified, serotonin-containing CGCs and buccal motoneurones were depressed in the presence of 2% halothane and blocked by 4% halothane. Similar synaptic depression has been demonstrated between the giant dopamine-containing neurone, R.Pe.D.1 and its follower cells (Winlow et al., 1981) in the presence of menthol (Haydon et al., 1982).

EFFECTS OF HALOTHANE ON EVOKED RESPONSES

Since neurones became silent at halothane concentrations of 1.0% or more, an investigation of the responses of **Lymnaea** neurones to injected depolarizing currents in the presence of halothane was undertaken. These experiments were carried out to further establish the concentration-dependent effects of halothane to evoked activity. Similar effects were induced by menthol (Haydon et al., 1982).

Depolarizing current pulses of 1 - 2 nA, amplitude and 5 s duration were injected into the soma of neurones. In normal saline these evoked a response which comprised a discharge of rapidly adapting action potentials in all cells, irrespective of whether they were previously quiescent or not. In the presence of halothane, the effect of the anaesthetic on evoked activity was concentration dependent (Fig. 1), and the evoked responses could be characterized on the basis of cell type (Fig. 2).

THE RELATIONSHIP BETWEEN NEURONE TOPOGRAPHY AND RESPONSES TO ANAESTHESIA

Our studies with halothane and menthol rapidly appeared to show that there was a correlation between the type of cell and its response to anaesthetics irrespective of the type of drug used. A further comparative study carried out with several different anaesthetic agents revealed important differences in the neuronal responses of individual, identifiable cells to applied inhalational and systemic anaesthetics.

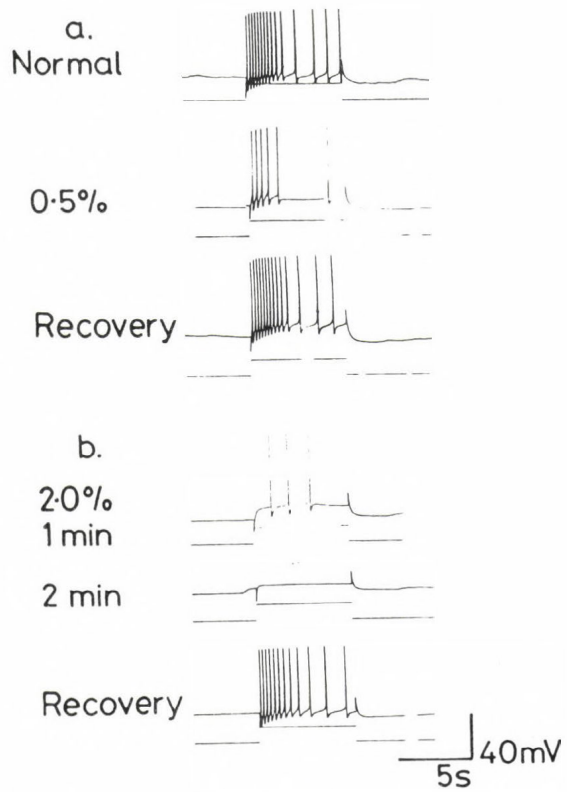

Fig. 1. The concentration dependence of the evoked response of RPD1 to 0.5 and 2.0% halothane, there was little effect on action potentials during the current pulse; i.e. the amplitude was maintained but there was a decrease in frequency (2.5 - 1.0 Hz). The higher concentration (2.00%) resulted in eventual loss of evoked activity but with no change, or very slight change, in membrane potential (Ca. 1-2 mV).

A series of specific cells types (R.Pe.D.1, VV1, R.P.D.1, A group, J cells, M group; Benjamin & Winlow, 1981) were studied with each drug. Although all agents had dramatic effects on neuronal activity within 20-30 seconds of being added to the bath, there were two major findings: (a) there was no apparent concentration dependence of the neuronal responses to enflurane or isoflurane; (b) with the exception of isoflurane, there was a correlation between each cell type and its response, irrespective of the drug used. This correlation consisted of either a gradual tendency towards quiescence or the occurrence of

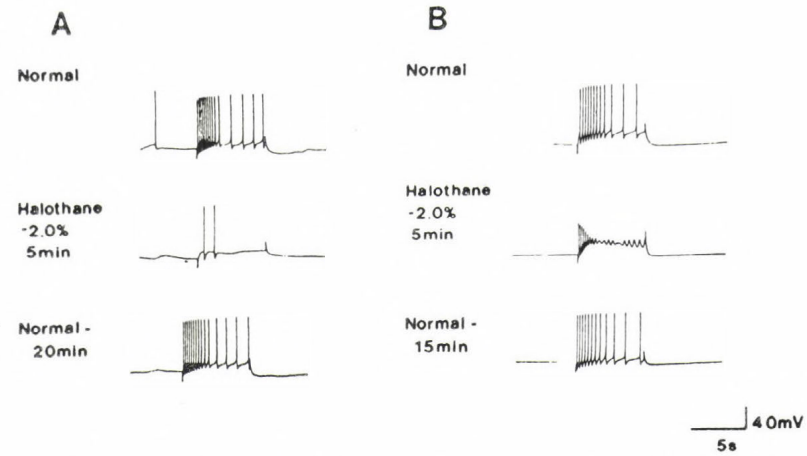

Fig. 2. The response of two different types of cell to 2.0% halothane. A) R.Pe.D.1 is a large, well characterized giant neurone (100 - 180 μm) of the right pedal ganglion (Slade et al., 1981) which has a characteristic firing pattern of regular spikes and normally receives considerable synaptic input (Benjamin & Winlow, 1981). In 2.00% halothane, the evoked activity of R.Pe.D.1 declined until quiescent with no detectable change in membrane potential. Through this decline in activity action potential amplitude and the AHP were maintained at their normal values; the major effect of halothane being the fall in frequency of action potentials during the pulse. The normal activity of the cell was restored 15 minutes after rinsing.
B) The effect of halothane on the evoked activity of A group neurones of the right parietal ganglion was quite different. These cells are much smaller than R.Pe.D.1 (in the region of 30-100 μm) and receive strong inputs from several different sources, including R.Pe.D.1, to which they are monosynaptically coupled (Winlow, Haydon & Benjamin, 1981). The evoked activity of the A group cell in halothane was characteristic of all the A group cells studied (n=8). The spike discharge was not maintained throughout the current pulse, but the activity declined into a series of membrane oscillations at a depolarized level. Higher concentrations of halothane (2.00%) eventually produced a quiescent state during which time action potentials could not be evoked.

spontaneous, paroxysmal depolarizing shifts (PDS) and oscillatory behaviour during evoked activity (Table 1). With isoflurane, all cells were immediately quiescent; the reason for this is not known. These results agree with previous observations with menthol (Haydon & Winlow, 1982) and halothane (Girdlestone, 1986).

PDS has been implicated in seizure activity such as epilepsy (Speckmann, Caspers & Janzen, 1972) and we suggest that it is an important mechanism in anaesthesia.

Table 1. Actions of anaesthetics on identified **Lymnaea** neurones

AGENT		PDS	Quiescence
Isoflurane	10	-	All cells
Enflurane	9	A, J, M, VV1	RPeD1, RPD1
Ketamine	6	A, J, M	RPeD1, RPD1
Thiopentone	6	A, J, M, VV1	RPeD1, RPD1
Pentobarbitone	3	A, M	RPD1

EFFECTS OF ANAESTHETICS ON NEURONAL MEMBRANES

Franks and Lieb (1987) recently suggested that anaesthetics act directly on membrane bound proteins which make up ion channels, as did Judge (1983). In many molluscan neurone somata, the inward currents of action potentials, are carried by both sodium and calcium ions. The pseudoplateau during repolarization is due to an inward calcium current (I_{Ca}) (Aldrich et al., 1979) and this inward current causes the post-burst hyperpolarization (PBH) by activating a calcium dependent potassium current (I_C) (Meech, 1978). Halothane, menthol and zero calcium abolish the pseudoplateau in many neurones (Haydon et al, 1982; Girdlestone, 1986) and reduce the amplitude of the PBH. These effects are consistent with the conjecture that anaesthetics block the inward calcium current. This hypothesis could account for the blockage of chemical synaptic transmission by anaesthetics.

CONCLUSIONS

Lymnaea is an excellent model system for the study of anaesthesia at both behavioural and cellular levels. Specimens of **Lymnaea** become anaesthetized well within the clinical range by halothane, enflurane and isoflurane. These anaesthetics modify action potential trajectory and block chemical synaptic transmission in a manner consistent with the suggestion that anaesthetics may block inward calcium currents by acting on membrane proteins.

In **Lymnaea** there is a correlation between a neurone and its response to anaesthetics irrespective of the type of drug used. These differences between cells presumably reflect differences in the protein ionophores of which their membranes are composed. Similar regional differences in neurones of the vertebrate brain may account for the differential effects of anaesthetics on different areas of the brain.

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DISCUSSION

LEAKE, L.: Using pyrethroids we have evidence that whether a pyrethroid induces PDS activity or repetitive activity depends on the balance of Na^+ and Ca^{2+} channels in a particular preparation. Do you know anything about the distribution of Na^+ and Ca^{2+} channels in your two different populations of cells?

WINLOW, W.: No, but we suspect that there are more calcium channels relating to sodium channels in those neurons which slowly become silent, as compared with those in which PDS is induced by anaesthetics. Voltage- and patch-clamp experiments are necessary to verify this finding.

LUKOWIAK, K.: You have used a very wide range of substances which produce similar effects. For example ketamine and halothane both produce PDS. Could you comment on this? And why do you think this is so?

WINLOW, W.: It is interesting and rather surprising that such a broad range of substances produce anaesthesia. There are many suggestions as to why this should be, but I favour the idea that these substances are somehow sequestered in "lacunae" within the membrane proteins. Once in position the properties of the membrane proteins are then modified by a macroconformational change in structure. Only molecules which fit the particular niches within the proteins can then act as anaesthetics. This explanation assumes that the anaesthetic agents pass through the lipid phase of the membrane in order to reach the membrane-bound channel or receptor proteins.

MAGAZANIK, L.: Did halothane change the cable properties of neuron?

WINLOW, W.: We do not have any evidence of the effects of halothane as yet, but we know that the unorthodox anaesthetic, menthol, blocks axon spikes of the dopamine cell RPeD1 (Haydon, Winlow and Holden, 1982).

ROUBOS, E.W.: It is well known that some anaesthetics exert profound long-term effects on the CNS (e.g. memory). Is it possible that halothane does act not only at the neural membrane but also at cytoplasmic sites (protein synthesis, DNA, etc.)?

WINLOW, W.: I think that it is almost certain that anaesthetic molecules such as halothane probably act at cytoplasmic sites within the cell, since these anaesthetics are freely soluble in lipids. It is therefore most probable that anaesthetics have a multiplicity of actions within the nervous system. Effects on protein synthesis are quite probable and could secondarily have actions on memory and membrane proteins in interesting and subtle long-term ways.

van der WILT, G.: The differential effects of anaesthetics on the activity of central neurons look very similar to those that we have observed as a result of central hypoxia. Prolonged ex-

posure of the CNS of Lymnaea to hypoxia results in complete suppression of spike activity in some neurons, while in other neurons paroxysmal depolarizing shifts are observed. Do you think some similarities might exist as to the mechanism of actions of anaesthetics and hypoxia on central neurons? (In vertebrates anaesthetics are known to interfere and to cause neural damage, due to central hypoxia.)

WINLOW, W.: A most interesting comment. Our preparations were fully oxygenated throughout our experiments. It is therefore interesting to speculate that hypoxic neurons might respond in a similar way to those undergoing anaesthesia. Perhaps hypoxia produces macromolecular changes in membrane proteins in the same way as we believe that anaesthetics act.

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MONOAMINE-DEPENDENT BEHAVIOURAL STATES IN THE PTEROPOD MOLLUSC CLIONE LIMACINA

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INTRODUCTION

It is widely recognized nowadays that bathing in solution of an individual transmitter substance can induce a patterned motor output of a neuronal system, or alter the patterned activity in a specific way. An immediate consequence of such findings is that the behavioural choice is fundamentally a chemical phenomenon. Conceptual consequences are less obvious.

Many authors still believe that it is possible to interpret transmitter-dependent behaviours in terms of the classical synaptic theory that rests on the assumption that neural circuitry is based on the anatomical connections of neurones. In fact, however, it makes no difference to this theory how many transmitters are involved.

Another school of thought makes a distinction between the secretory terminals destined for classical transmission, on the one hand, and those responsible for overall modulation, on the other (see e.g. Sombati and Hoyle 1984, Arshavsky et al. 1987). The diversity of neurotransmitters is a more meaningful parameter for this view than for the classical theory.

In our opinion, the phenomenon of transmitter-dependent behaviour is a major challenge to the theory of integration based on specificity of anatomical connections. An alternative would be the integration based on chemical selectivity. In terms of patterned activity, the repertory of an idealized heterochemical neuronal system would then correspond to the diversity of transmitters: one pattern, one transmitter.

To explore the alternative explanations, convenient types of preparation are needed. We wish to demonstrate here that the neural machinery of Clione may represent a favourable system for studying the integrative function of transmitters.

BEHAVIOURAL STATES

Pteropod molluscs are amongst the most active of invertebrates. The biology and neuroanatomy of C. limacina was excellently described by Nikolai Wagner of St.Petersburg (I885). Interestingly, this author suggested (a century ago!) the use of Clione as a model animal in an attempt to understand how the nervous system works. (On normal behaviour of Clione, see also Litvinova and Orlovsky I985, Satterlie et al. I985, Sakharov and Kabotyanski I986).

Clione has a spindle-shaped body provided with a pair of fins (wings). There are two sorts of locomotion in Clione: slow (vertical) and fast (horizontal) swimming. In both the wings move synchronously.

During slow swimming, the animal moves upwards with a vertical body orientation and forward direction of paired anterior tentacles. Tactile stimulation of the tail makes the animal swim faster whereas when the anterior tentacles contact the surface, they retract, swimming ceases for several seconds, and the animal sinks. Tactile stimulation of the head or a wing results in a similar swimming arrest and local retraction. Inhibitory episodes may also occur spontaneously.

Besides short inhibitory episodes, there occur long lasting periods of rest when the animals can be seen lying on the bottom of an aquarium for minutes or even hours. In a resting animal, the body is shortened, the wings and tentacles are retracted. There is no active escape in response to a single tactile stimulation of the tail. Instead, a tactile stimulus induces local retraction or general bending of the body.

The fast, horizontal swimming, unlike the slow one, is not interrupted by periods of rest. In the White Sea, this sort of locomotion is generally utilized by the animals in spring and early summer when Clione feed on another pteropod, Lima-

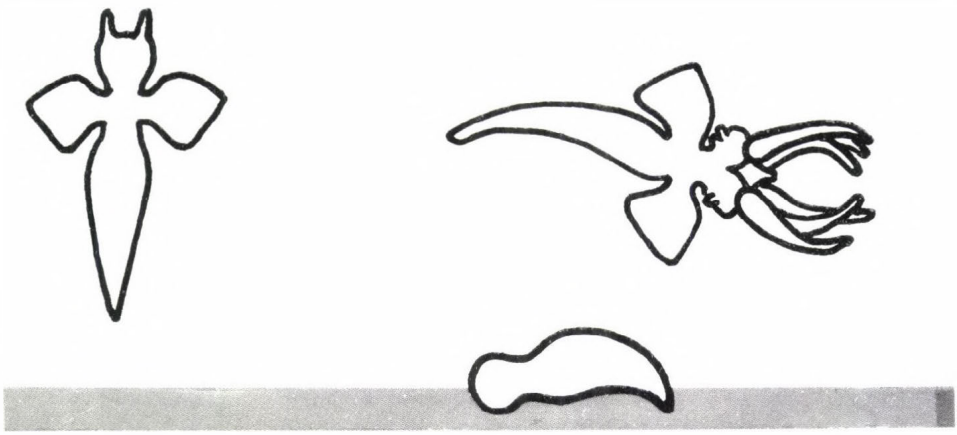

Fig. 1. Normal behavioural states in Clione: slow swimming (left), feeding arousal (upper right) and resting (lower right).

cina. Contact with the prey results in an explosive extrusion of three pairs of adhesive buccal appendages, the cephaloconi. In Clione aroused by the presence of the prey, rapid reversible extrusions of these adhesive cones can occur spontaneously and continue for an extended period of time during fast swimming. Both fast swimming and cephaloconi movements are thus components of an arousal associated with initial steps of feeding behaviour.

To summarize, at least three distinct behavioural states can be recognized in Clione, viz. slow swimming, resting, and feeding arousal (Fig. 1). It seems natural to consider slow swimming as a background state, a sort of starting-point from which an animal can change to either rest or predation. We have found that this background state can be changed into another by a single injection of a transmitter monoamine or its metabolic precursor.

Injection of dopamine (DA, 2 µg/g, dissolved in sea water containing 50 µM ascorbic acid) resulted in swim inhibition associated with augmented retraction. The effects characteristic of DA could be studied on a slower time scale when the animals were injected with the DA precursor, L-DOPA (200 µg/g). There occurred progressive extension of inhibitory episodes and, finally, swimming ceased altogether. The animal was

lying on the bottom, its body shortened and appendages retracted. The slightest tactile stimulus evoked local movements or general bending. Similar movements also occurred spontaneously. These effects of DOPA reached maximum after several hours, then decreased, to disappear in about two days.

With serotonin (5HT) injection, 20-40 µg/g, fast swimming immediately developed and then, several minutes later, explosions of adhesive cones began. The anterior tentacles were moved towards the back of the head while the proboscis and radula moved forward. Swim inhibition and retraction to tactile stimulation were highly reduced. At the maximum of the 5HT effect any tactile stimulus could release extrusion of adhesive cones. 5-Hydroxytryptophan (5HTP) acted similarly though much more slowly. Notably, the effects of 5HTP (as well as those of DOPA) were temperature-dependent. For example after injection of 100 µg/g 5HTP, extrusion of cones was first observed in 10-20 h at 6^{o}, in 5-15 h at 10.5^{o}, and in 1.5-2.5 h at 13^{o}C. This indicates that the effects were due to product(s) of metabolism rather than to the injected drugs themselves.

DA and 5HT thus produce opposite effects on the behavioural state, the former amine shifting behaviour to a state of rest and the latter to a state of arousal. At the cellular level, 5HT has been shown to antagonise a specific type of DAergic transmission: in this respect, 5HT resembles ergometrine, ergotamine and related ergots (Shozushima 1984). We investigated, therefore, the effects of these ergots on Clione behaviour. We found that bathing Clione in sea water containing ergometrine altered behaviour in a 5HT-like manner. The following components of the 5HT-dependent behaviour were observed: a reduction in the duration of the spontaneous inhibition of swimming (from 0.001 mg/l) up to complete disappearance of swim arrests (at about 0.01 mg/l), disappearance of swim inhibition and retraction in response to tactile stimulation (from 0.01 mg/l), appearance of spontaneous extrusion of adhesive cones (from 0.05 mg/l). Wing movements were highly regular and strong, the animals were demonstrating a ceaseless stereotyped swimming for hours or even days. The

frequency of the wing beat was, however, low as compared with that in animals exhibiting fast swimming induced by 5HT. Moreover, it was lower than the initial frequency observed during slow swimming prior to the ergot action. A decrease in frequency was obviously due to a delay at a specific phase of the locomotor cycle, viz. when the wings are in their most ventral position. It appears that there exists an ergometrine sensitive link in the neural machinery of the rhythm generation. Injection of ergometrine produced a similar effect. Ergotamine and methylergometrine altered the Clione behaviour in a similar manner. For further details see Sakharov and Kabotyanski (1986). It seems possible that ergots block an inhibitory receptor for DA and tilt the balance of two antagonistic amines, DA and 5HT, in favour of 5HT. As a result the 5HT-dependent behavioural state appears.

NEUROPHYSIOLOGICAL CORRELATES

Thus, there appears to be a monoaminergic control of behavioural states based on the balance between DA and 5HT. The next step is to correlate these phenomena with the use of isolated preparations. In principle various components of overall behaviour could be used for this study. The fictitious swimming seems to offer an especially convenient reference system for monitoring behavioural states. The obvious advantages are as follows. (1) Swimming is expressed differently at each of the three states. (2) Unlike in other swimming gastropodes, in Clione the swimming rhythm is easily generated using isolated preparations (Sakharov 1960, Arshavsky et al. 1982). (3) Cellular mechanisms for generation of both slow and fast swimming have been thoroughly described (Arshavsky et al. 1985). Due to the latter study, several identifiable neurones are fully characterized and can be used as reference cells.

We used the preparation of isolated pedal ganglia and recorded mainly from two large paired output neurones of the central pattern generator for swimming, cells 1A and 2A. Activity of these antagonistic neurones is revealed in opposite

phases, cell 1A being a wing elevator and cell 2A a wing depressor (see also Satterlie and Spencer I985). The preparation and electrophysiological technique was as described by Arshavsky et al. (I985) with the exception that we did not embed ganglia in agar. Animals were dissected in a 1:1 mixture of sea water and isotonic $MgCl_2$.

We confirmed previous findings (Arshavski et al. I982, Satterlie and Spencer I985) that generation of a swimming rhythm is maintained for many hours in isolated pedal ganglia. In our preparations, this fictitious swimming was usually interrupted by spontaneous pauses, as is the normal swimming. Effects of drugs upon such intermittent fictitious swimming turned out to be similar to their effects on actual swimming and its periods of arrest.

Bathing isolated pedal ganglia in ergometrine (Fig. 2) or methylergometrine abolished spontaneous arrests of swimming. (Note that electrical activity of swim neurones was recorded at a low speed. Each spike-like depolarization corresponds in Fig.2A and 2C to a whole phase in the swim cycle,and is represented by a prolonged EPSP accompanied or not accompanied by a burst of action potentials.) The swimming rhythm was slightly slowed by these ergots in isolated preparations, as it was in whole animals. Spontaneous arrests of fictitious swimming were also abolished by adding 5HT or, with a larger latency, 5HTP to the bath. Effects of both 5HT and 5HTP included an increased production of action potentials per cycle, and a characteristic change in the form of the patterned electrical activity underlying wing movements, as it is shown in Fig. 3. This change in form represents, according to Arshavsky and coworkers, switch from the slow swim to the fast one.

When a preparation did not reveal spontaneous inhibition of swimming, inhibitory episodes could be initiated by adding DA to the bath.

It seems therefore that the main pharmacological effects revealed in behavioural experiments and described in the preceding paragraph have their correlates associated with a greatly reduced neurophysiological preparation.

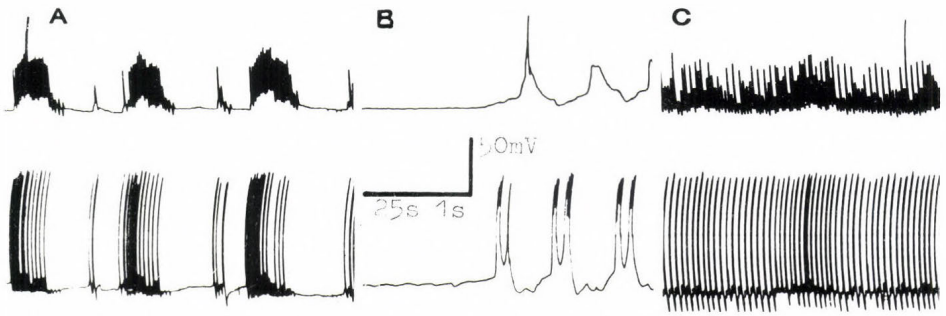

Fig. 2. Effect of ergometrine on intermittent fictitious swimming. Simultaneous intracellular records from cell 1A (lower) and 2A (upper). A, Intermittent swimming pattern prior to bathing in ergometrine. B, The same, at a faster speed. C, Continuous swimming, 11 min bathing in 0.02 µg/l ergometrine maleate.

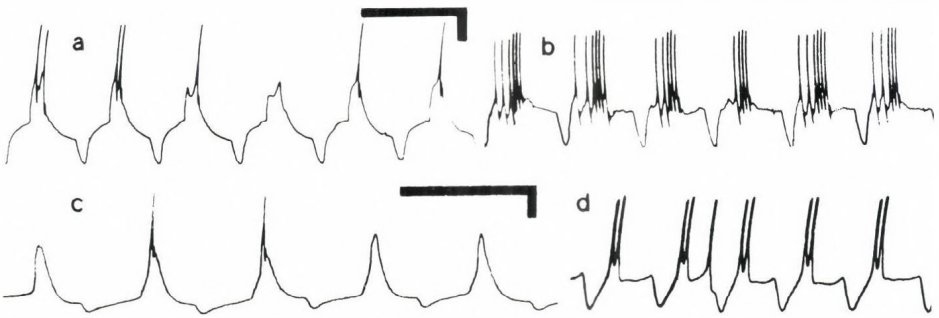

Fig. 3. Similarity in effects of 5HT (20 µg/ml, upper record) and 5HTP (200 µg/ml, 45 min, lower record). In both experiments, the swim rhythm of cell 2A is shown prior to (a, c) and after action of the drug (b, d). Scale, 1 s, 20 mV.

MONOAMINE-CONTAINING NEURONES

The aim of the last part of the study reported here was to map the distribution of monoamine (MA)-producing neurones that might participate in the mechanisms underlying MA-dependent behaviours. We used a glyoxylic acid histochemical method (Torre and Surgeon 1976) and a sensitive modification for use in both sections and whole mounts (Kabotyanski 1985). Microspectrofluorimetry was as described earlier (Sakharov and Salimova 1980). A recently developed methodological com-

bination of fluorescence cytochemistry and electrophysiology (Sakharov and Kabotyanski 1987) was also used. The branching pattern of neurones was investigated using ionophoretical injection of Lucifer Yellow from a glass microelectrode. Besides the isolated pedal ganglia preparation, we used in this part of the study a preparation,consisting of all central ganglia, and preparations, consisting of the CNS and an innervated portion of the periphery, e.g. the tail.

In general, glyoxylate treatment induces in all molluscs so far investigated two clearly distinct colours of fluorescence, yellow of 5HT and blue-green of CAs. Our results obtained in late summer and autumn, when Clione exhibit slow swimming, were in sharp contrast to this situation. No yellow fluorescing neurones were found in the central and peripheral nervous system of Clione (Figs 4, 5).

In the CNS, green-fluorescing (CA-containing) cell bodies were found only in cerebral, buccal and pedal ganglia. Their number was small as compared with that found in other gastropods. Seven paired, individually identifiable CA neurones were located in the cerebral ganglia (Fig. 4). These newly identified cells were each assigned an identification number that corresponds to their position (C, cerebral) and CAergic nature (e.g. RCCa5, Right Cerebral CA cell 5). The cells CCa1 and CCa2 located near the exit of the cerebro-pleural connective are notable due to their relatively large size and bright fluorescence. The cell CCa1 has an axon that runs towards the contralateral cerebral ganglion where it divides to supply with its branches the sheath of nerves and connectives that leave the ganglion. Two symmetrical cells CCa1 were shown to be electrically coupled (Fig. 6). Injection of Lucifer Yellow into one of these cells resulted in the appearance of the dye in the cell body of the other provided that current was running between the injecting electrode and the electrode inserted into the paired cell. In contrast, in cells CCa2 a bilateral excitatory chemical connection was revealed. Each cell sends its axon into the posterior portion of the body. Mechanical stimulation of the tail produced afferent activity in these paired neurones, their sensory fields partially

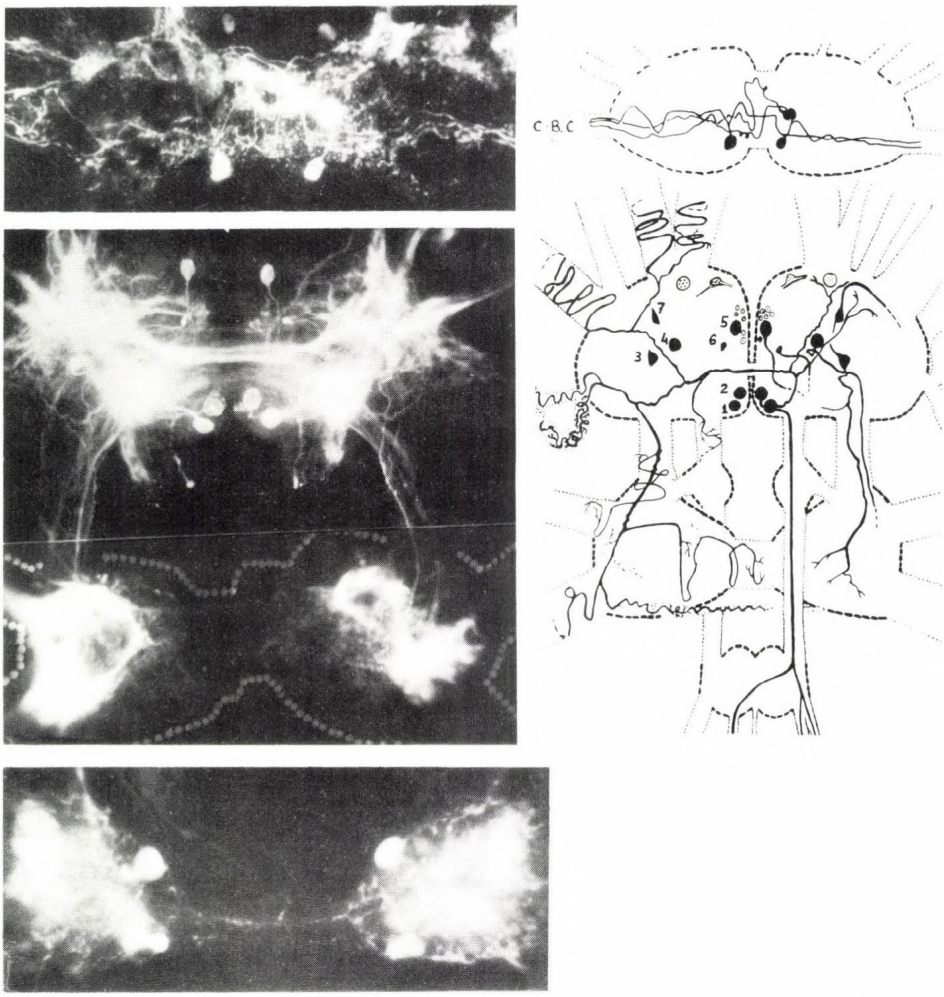

Fig. 4. Fluorescence histochemistry of monoamines in the CNS. Left, from top, Buccal, cerebral and pedal ganglia of a slow swimmer (only green fluorescence is seen in photomicrographs of whole mounts). Left, lower, Pedal ganglia of an animal aroused with a 5HTP injection (yellow fluorescence can be seen in large symmetrical cell bodies). Right, A schematic representation of green (blackened) and yellow (dotted) fluorescing neurones in the cerebral and buccal ganglia.

overlap. It appears that cells CCa2 (and maybe CCa1) may have a mechanosensory function. The cells CCa3 and CCa4 are of particular interest in that their axons supply the pedal ganglia. In the buccal ganglia, there were only three green-fluorescing

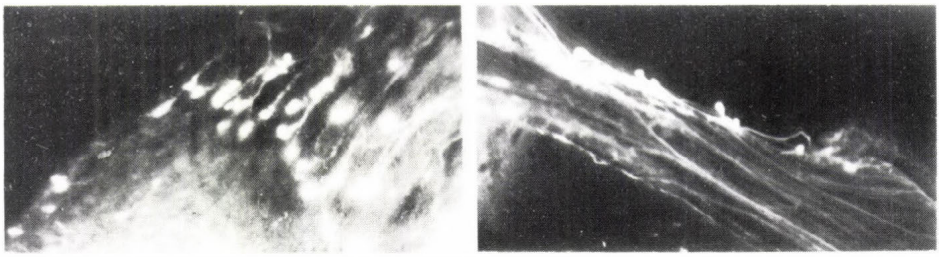

Fig. 5. Green fluorescing neurones in the anterior oesophagus (left) and at the base of the penis (right).

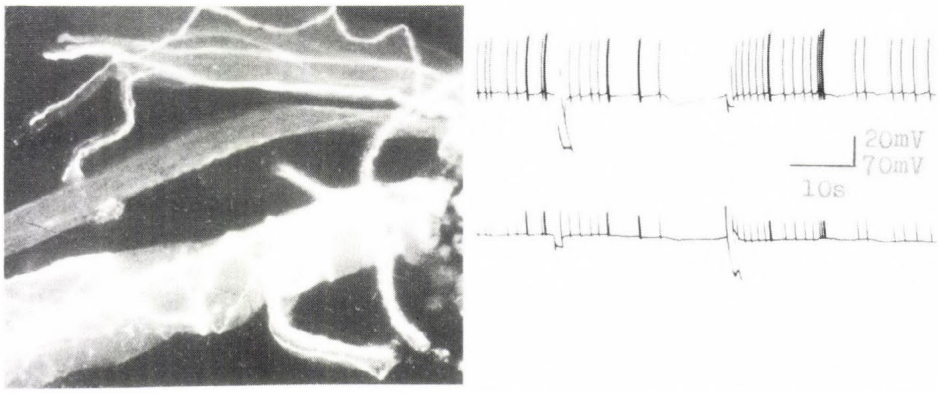

Fig. 6. Cells CCa1: branching of the CCa1 axon in the sheath of contralateral cerebral nerves as seen in a whole mount preparation treated with glyoxylate (left), and electrical coupling between two synchronously active CCa1 cells (right).

neurones, one paired and one unpaired (Fig. 4). CA cells of the pedal ganglia were found to be small and variable in both number and locations.

The neuropile of all ganglia contained many green fibres most of which appeared to enter from the periphery. Indeed, numerous green cells were found outside the CNS, mainly in the anterior oesophagus, at the base of the penis (Fig. 5) and in the epidermis. The peripheral CA neurones could be seen sending their proximal processes to nerves, going to the CNS. All green cells so far investigated gave an emission peak at 483-485 nm (Fig. 7A).

In animals treated with 5HTP and emitting feeding arousal, the picture of MA fluorescence was very different. Two

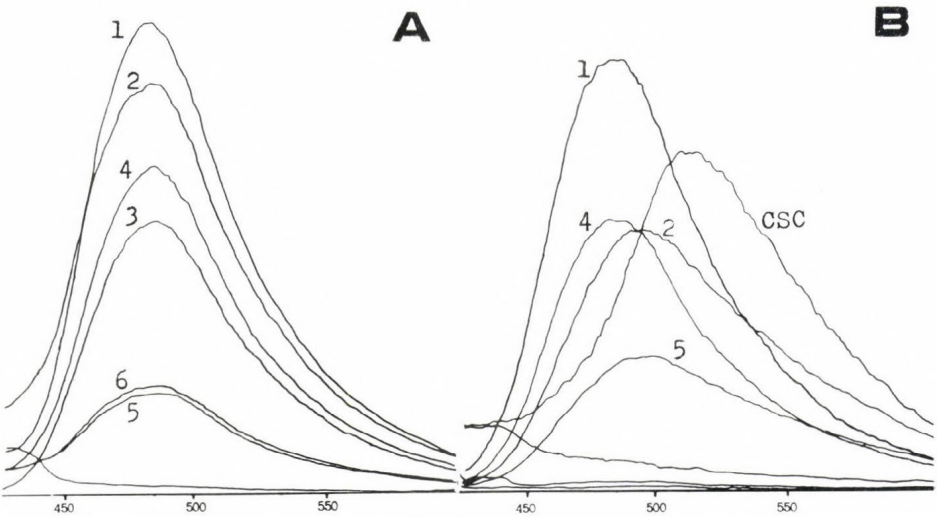

Fig. 7. Emission spectra of fluorophores in identifiable MA neurones of a slow swimmer (A) and an animal aroused with 5HTP (B). The glyoxylate method. CSC, Cerebral serotonin cell. Note a shift in spectra of CCa2 and CCa5 neurones.

large symmetrical yellow cells, besides two groups of small neurones, were seen in each cerebral ganglion. One additional pair appeared in the buccal ganglia. The pedal ganglia contained several large yellow neurones located at the medial aspect of each ganglion (Fig. 4). These pedal 5HT neurones have been investigated electrophysiologically. They revealed enhanced activity during swimming periods. No direct effects of pedal 5HT neurones on fictitious swimming could be found.

In the peripheral nervous system of 5HTP-treated Clione numerous yellow fluorescing fibres were found. The wall of the adhesive cones was also rich in yellow perikarya.

The newly appeared yellow cells of the CNS had emission maxima at about 512-514 nm. This figure is characteristic of a 5HT derivative. However, one striking finding did emerge. Cells CCa2 and CCa5 exhibited a shift in the emission peak from the "normal" 485 nm to 494 nm (Fig. 7B). It appears that CA neurones differ in their ability to take up (and perhaps to utilize) 5HTP.

CONCLUSION

Although the cellular analysis of MA-dependent behavioural choice in Clione is still in its initial stages, several preliminary conclusions can be drawn. The preparations used appear to be advantageous for such an analysis because neurophysiological correlates of the behavioural states are available, and MAergic neurones are few in number and, at least partially, individual and identifiable. An obvious disadvantage is their relatively small size. To overcome this problem, the newly developed technique of glyoxylate-filled microelectrode (Sakharov and Kabotyanski 1987) has proved helpful.

The most obvious difference between the distribution of MAs in Clione and other gastropods is the absence of the 5HT fluorescence in the normal slow swimmers that we dealt with. We suppose that the low level of 5HT is a seasonal feature of the Clione nervous system, and is characteristic of animals which do not exhibit predatory behaviour. The 5HT fluorescence appeared in neurones of Clione exhibiting a persistent feeding arousal due to the artificial elevation of the 5HT level in the organism. From these observations it seems possible that the seasonal arousal is similarly associated with an increase in the 5HT content.

Our findings indicate that CA neurones of Clione are, at least partially, mechanosensory cells. This finding confirms earlier indirect evidence that molluscan mechanosensory neurones have a DAergic nature (Sakharov and Salánki 1980). In Clione, the DAergic neurones appear to comprise a major inhibitory input to the neuronal machinery of locomotion. When the DA production was increased, the behaviour was shifted towards that characteristic of the resting state. On the contrary, when the DAergic inhibitory input was blocked, a stereotyped disinhibited swimming was established, and the animals emitted the prey capturing behaviour, which was similar to that induced by 5HT. This implies that the choice between the three behavioural states depends mainly on the balance between the two transmitter amines. It appears to be the overall MAergic situation that matters, not the "chemical signal-

ling" in the traditional sense. This suggestion deserves further consideration. Anyway, the results obtained in this study are consistent with the view that the integrative function of the nervous system can be understood in terms of the diversity of neurotransmitters (Sakharov 1983, 1985), and provide a basis for a more detailed investigation of transmitter-dependent behaviours in Clione.

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DISCUSSION

HONEGGER, H.W.: Did you record from the motor neurons while Clione could move?

SAKHAROV, D.A.: No, we did not record from nerve cells in a moving animal. Simultaneous recording of electrical activity of motor neurons and movements of wings had been obtained earlier by Arshavsky et al., and we could just use their map of identifiable and characterized elevator and depressor neurons.

K. LUKOWIAK: I would just like to comment that Rich Satterlie is now working on a species of Climia at Friday Harbour in Washington and has found I believe some peptidergic neurons which may exert some control over swimming behaviour.

SAKHAROV, D.A.: Thank you for this comment. Satterlie and coworkers published in 1985 results obtained with the same species of Clione, which is used in my country, Clione limacina. This species is rather numerous in both the North Atlantic and North Pacific oceans. Regretfully, many findings of the Friday Harbour group just repeated what had been published earlier in Russian. Only one example. They described a swimming preparation consisting only of pedal ganglia and the two wings. Such

a preparation maintains swimming for many hours. We use it for many years in practical studies of students at the Marine Biological Station of the Lomonosov University. It was my fault that I described this preparation only in Russian in my paper which appeared in 1960. It would be wise of those who are involved in studies of Clione to be in closer contact and to coordinate our work. As far as I know, peptidergic neurons of Clione are not currently investigated in the Soviet Union.

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SEROTONERGIC CONTROL OF CILIARY LOCOMOTION IN A TURBELLARIAN FLATWORM

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INTRODUCTION

In gastropod molluscs and leeches, serotonin (5-hydroxytryptamine, 5-HT) initiates or enhances locomotion and associated behaviours, such as feeding and hunting (see Kabotyanski and Sakharov, Leake and Woodward, Syed et al.; this volume). The locomotor activity of lower vertebrates appears to be similarly correlated with the level of 5-HT in the CNS (Fingerman 1976, Genot et al. 1984). These animals represent divergent zoological groups and utilize different mechanisms for body movement. Why then do they share a common chemical way of achieving arousal? A working hypothesis might be that 5-HT-ergic control of locomotion is a phylogenetically conservative feature inherited by advanced phyla from a common ancestor.

This hypothesis cannot be tested directly. Primitive features of ancestral organization can, however, be found and investigated in living representatives of ancient zoological groups. We present here the results of our study of the planarian Dendrocoelum lacteum. The ciliary locomotion of turbellarian flatworms is generally believed to be of primitive rather than of secondary character.

Not long ago, it was widely assumed that the nervous control of ciliary activity is not developed in planarians. This interpretation of early findings (Alverdes 1922) was adopted in authoritative texts. Correspondingly, the nervous endings located in or near the planarian ciliated epithelium were described as sensory terminals. An alternative view has been con-

sidered by Welsh and Williams (1970) inspired by observation that ciliary motion progressively weakens in planarians exposed to reserpine. Using the Falck-Hillarp fluorescence method, they have found that indolamine-containing neurones predominate in the ventral region of the body and innervate the ventral epithelium. Welsh and Williams have suggested that these 5-HT cells "may be sensory or they may have a cilioregulatory role". The latter idea receives support from the results of the present study.

EXPERIMENTAL PROCEDURES

Adult specimens of Dendrocoelum lacteum were used. Prior to killing the animals were anaesthetized and relaxed by immersing them in 0.15-0.3 M aqueous $CoCl_2$ solution, as described earlier (Sakharov 1972).

Measures of locomotor activity

To analyse the effect of the metabolic precursor of 5-HT, 5-hydroxytryptophane (5-HTP), on locomotion, groups of 5 animals were put into Petri dishes filled with 45 ml of aqueous 5-HTP solutions of various concentrations at 19°C. The experiment was repeated 5 times using new animals. We periodically counted how many times the animals crossed a line dividing the bottom of the dish into two equal parts.

Immunocytochemistry

A monoclonal antibody to 5-HT conjugate was obtained from Serotec (product code MCA 76A, batch C23). It was used diluted to 1:1000 and 1:2000. Flatworms were fixed by immersion for 12-18 h in cold paraformaldehyde, depolymerized in 0.1 M phosphate buffer. Frontal or sagittal sections were cut at 10 to 20 µm and placed on glass slides. The staining procedure involved treatment with 0.3% Triton X-100 in 0.1 M PBS, pH 7.4, for 1-2 h, washing three times, 15 min each, in PBS solution containing 0.1% (w/v) bovine serum albumin, addition of anti-

body diluted in PBS with 1% normal BSA, incubation overnight at $4^{o}C$, washing three times, addition of rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase, incubation for 1 h at room temperature, washing three times, addition of 3,3-diaminobenzidine freshly diluted in 0.05 M Tris-HCl buffer, incubation with 0.01% H_2O_2 for 15 min. The reaction was stopped by adding water. Sections were then dehydrated in graded concentrations of ethanol, clarified in xylene, and mounted in Canadian balsam. Control sections were treated with the following changes in the procedure: (1) the primary antibody was replaced by a monoclonal antibody to vimentin, (2) the primary antibody was excluded altogether, and (3) both the primary and the secondary antibodies were excluded.

Electron microscopy

Pieces of tissue of the middle part of the body were fixed for 4 h in cold 2.5% glutaraldehyde buffered to pH 7.4 with 0.05 M sodium cacodylate, and postfixed for 1 h in 2% osmium tetroxide. The blocks were then dehydrated in graded concentrations of ethanol and embedded in standard resins. Ultrathin sections were stained with uranyl acetate and lead citrate. They were examined with an EMV-100 LM electron microscope. For more details see Sakharov et al. (1986).

Neurotoxins

For better ultrastructural discrimination between different types of neurones, some animals were treated with the toxic analogue of 5-HT, 5,6-dihydroxytryptamine (5,6-DHT) or with that for dopamine and related catecholamines, 6-hydroxydopamine (6-OHDA). Treatment was by immersing the worms for 30 min into a freshly prepared 25 mg/l aqueous solution of the toxin containing 0.1% ascorbic acid as an antioxidant. Control animals were kept in the solution of ascorbic acid. As soon as the 30 min period of treatment was over, one group of animals and the control ones were taken for tissue fixation. Other groups were fixed at intervals. Both stained and unstained ultrathin sections were examined in this series.

Substances were obtained from Calbiochem (5-HTP), Sigma (5,6-DHT) and Serva (OHDA).

RESULTS

Typically for flatworms, planarians have a thin leaf-like body. D. lacteum reaches a length of 20-25 mm and is covered with cilia on its ventral surface. The mouth is ventral in the middle and the pharynx is everted through it during feeding. D. lacteum is entirely carnivorous. It actively hunts by gliding on the substrate and reacting to prey stimuli (Herrmann 1984). The microscopic anatomy of D. lacteum has been described by Iijima (1884) and von Gelei (1912).

Locomotor behaviour

Like other freshwater triclads (Pearl 1903), Dendrocoelum does not swim, it moves exclusively over solid substrates and has two sorts of locomotion: gliding and crawling. Gliding is brought about by the action of the ventral cilia which beat in the mucus secreted by unicellular glands. In the gliding animal, the head is raised from the substrate and does not take part in the locomotor movements. There are well-marked alternations of gliding activity and rest. When the animal passes from the resting condition into gliding its body lengthens and flattens. Crawling is a purely muscular movement. It is of less frequent occurrence than gliding. To induce crawling, we stimulated the posterior end of the worm with a stiff hair. Under this condition, the animal abandoned ciliary locomotion and released a series of rapid leech-like steps (6-7 cycles, at most). This fixed-action pattern occurs only as a temporary escape mechanism. Crawling can be also released with drying, the action of light and other strong stimuli. As soon as muscular steps are over, the animal returns to the gliding mode of locomotion.

Motor behaviour was dramatically changed in animals kept in an appropriate 5-HTP solution. Quantitative changes are repre-

Table 1. Effects of 5-HTP on locomotor activity of D. lacteum. Each figure represents pooled activity of 25 animals (in arbitrary units)

5-HTP mg/l	Hours of treatment														
	1	2	4	5	7	8	9	10	11	12	14	15	19	24	48
0	7	10	12	3	0	2	4	0	7	2	0	0	4	3	4
25	12	10	11	3	5	2	6	3	4	0	9	6	12	14	6
50	15	5	4	3	2	5	13	20	16	16	21	32	34	31	27
100	8	5	14	14	19	21	42	44	44	46	48	50	48	50	49
200	31	21	22	24	43	68	60	81	98	103	109	101	104	98	99

sented in Table 1. The dependence of motor activity on 5-HTP concentration is convincingly expressed in the Table. The lowest concentration, 25 mg/l, seems to be close to the threshold because its effect is hardly noticeable. An initial rise of activity demonstrated by all groups may represent a response to a new environment. Then follows a sustained rise of motor activity and, finally, a concentration-dependent plateau level. In the highest concentration, 200 mg/l, the figures may be underestimated because the animals had difficulty in making proper contact with the substrate. This appears to be due to an excessive secretion of mucus.

Qualitatively, the effects of 5-HTP were also impressive. At an advanced stage of the action of the drug, the periods of rest were totally abolished in all animals kept in 50 mg/l and above. The animals were invariably flattened and lengthened and they glided over the bottom of the dish for several days without coming to a perceptible stop. Under these circumstances the animals were never seen to crawl. This implies that cilia showed continuous beating in treated animals, whilst muscular locomotion appeared to be completely inhibited and crawling could not be induced even by a strong stimulus. Our preliminary observations indicate that the feeding activity is higher in animals treated with 5-HTP than in the controls.

Distribution of 5-HT immunoreactivity

The nervous system of Dendrocoelum consists of two head ganglia, two ventral cords, and several compartments of the so-called peripheral nervous system. In the following survey of our immunocytochemical results we shall refer to Baguna and Ballester (1978) who reexamined the planarian nervous system using neurohistological techniques.

Control cryostat sections (Controls 1 and 2, see Experimental Procedures) and the sections incubated with antibody to 5-HT had certain features in common, but differed in others. In both instances, some unicellular glands were intensively stained. They seem to correspond to mucous and salivary glands described by Iijima (1884). This cross-reactivity should be kept in mind to distinguish the non-specific glandular staining, as that seen in Fig. 1C, from the specific staining of 5-HT-containing nerve cells and fibres. In Fig. 1A another sort of non-specific staining can be seen in the alimentary system. This staining is due to the peroxidase activity of the food, Tubifex.

We found 5-HT immunoreactivity in some parts of the nervous system but could not find it in others. No staining was detected in the gastrodermal plexus or the genital organs. Only a few neurones were labelled in the region of paired head ganglia, and the "brain" could not therefore be seen as a distinct structure. Among internal organs, the pharynx is notable for its fairly rich 5-HTergic innervation: radial fibres derived from the inner cylindrical nerve net of the pharynx could be traced to its inner (ciliated) and outer surfaces.

The darkest immunostaining was found in the submuscular plexus (Fig. 1). As Baguna and Ballester have put it, the submuscular plexus revealed by conventional neurohistological methods is fairly uniformly distributed over the body and is well developed in the cephalic region. The immunostained submuscular plexus was, however, far from being uniformly distributed. It occurred only in the ventral part of the animal with the exception of the head (Fig. 1A-C).

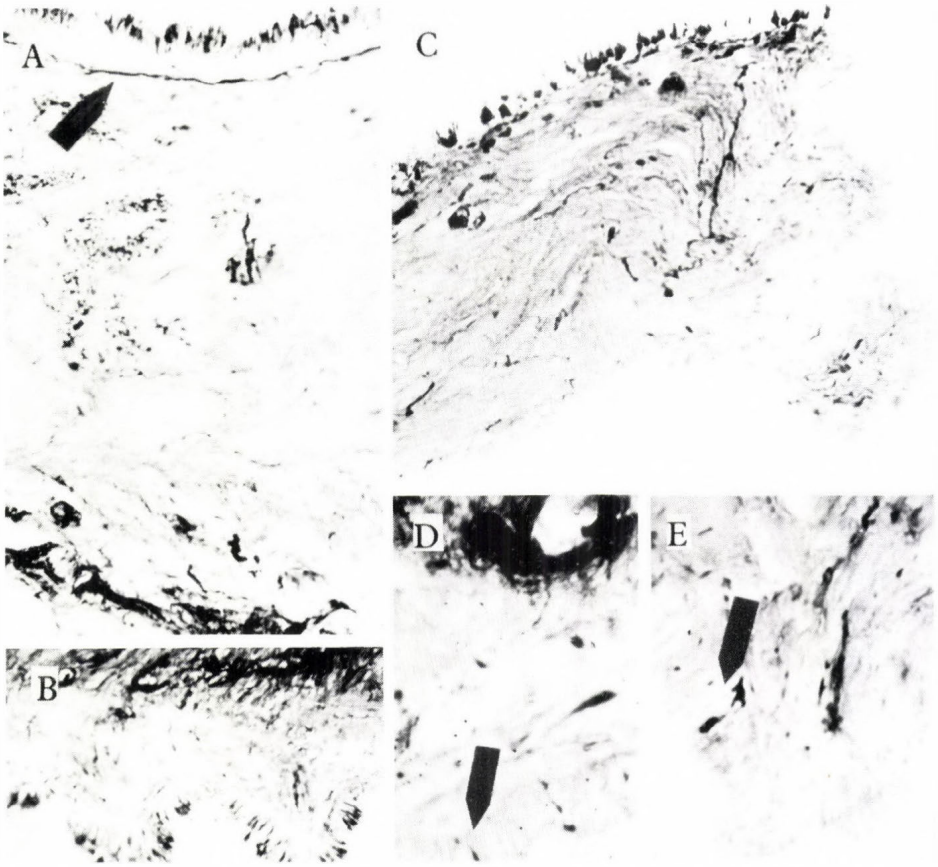

Fig. 1. 5-HT-immunoreactivity in sagittal cryostat sections. In A and B the sections have been additionally stained with haematoxylin and eosin to visualize rhabdoids in the dorsal (A) and ventral (B) epithelium. Dorsal surface is uppermost. A – Dorsal part of the body wall and the underlying parenchyma. Axonal staining in the dorsal musculature (arrow). The densely stained ventral submuscular plexus is seen in the lower part of the micrograph. B – Ventral side of the body wall. C – Head. D and E – Ventral part of the body at higher magnifications. Intraepithelial immunoreactive terminals (arrows).

Throughout the body, single stained neurites could be seen spreading from the ventral submuscular plexus in various directions. Scattered fibres traversed the head parenchyma (Fig. 1C). Small bundles were occasionally seen running up between parenchymal cells of the body. The longitudinally oriented

stained axons innervating the muscles of the dorsal part of the body wall (Fig. 1A) appear to derive from these ascending bundles. At the dorsal aspect of the animal stained fibres go no further than the muscular layer. On the ventral side the situation is different. The fibres derived from the ventral submuscular plexus were rather numerous here. They were generally very thin and formed delicate bundles running towards the epithelium (Fig. 1B). Immunostaining did not reveal the network of fibres disposed in a single plane and described by Baguna and Ballester as the subepithelial plexus. Instead, ramifying stained fibres had a predominantly transverse orientation. Here and there very thin nerve endings could be seen entering the epithelium and running up between the ciliated cells (Fig. 1D,E). Intimate association of 5-HT-containing nerve endings with the epithelial cells occurs throughout the ventral side of the body, except the head.

Neurociliary synapses

Under electron microscope, the ventral epithelium appears as a monolayer of columnar ciliated cells. The underlying basement membrane consists of a thin, dense basal lamina and a fibrous reticular lamina facting circular muscles. Two types of secretory axon terminals could be seen at or in the epithelium. Type I terminals lie between the fibrous lamina and muscle cells. They are axon enlargements containing clusters of clear vesicles with diameters 80 to 90 nm. The clusters usually face the basement membrane (Fig. 2A), i.e. the ciliated epithelium. Type II terminals are very thin neurites which cross the basement membrane and enter the epithelium. They form small bundles consisting of only two to four axons. These axon terminals have uniform appearance; they are characterized by numerous neurotubules and by vesicles of variable density. Some of the vesicles are clear whilst others, in the range of 70-100 nm, contain a dense core (Fig. 2B). Type I terminals seem to correspond in their position to the subepithelial plexus, while Type II terminals resemble the 5-HT immunoreactive fibres. We interpret their contacts with ciliated cells as two types of neuro-

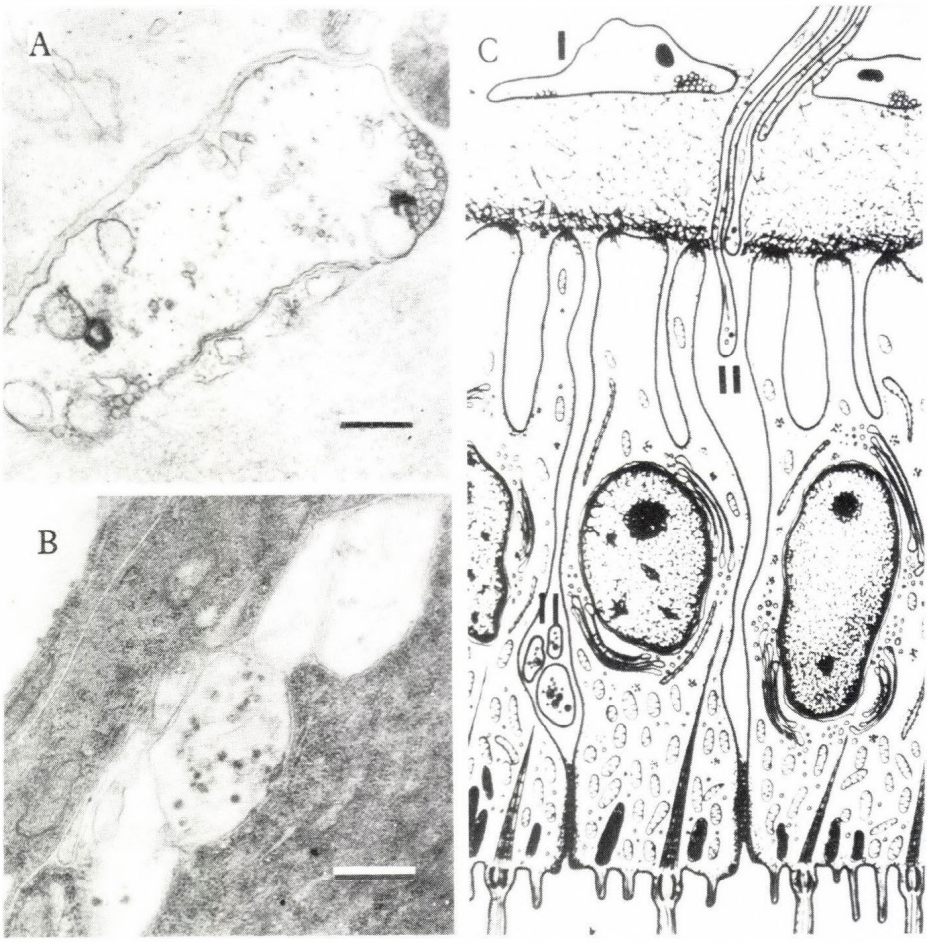

Fig. 2. Two types of axons terminating near ciliated cells. A – Type I terminal. Scale 0.5 µm. B – Type II terminal. C – Schematic representation of the innervation pattern in the ventral epithelium.

ciliary synapses (the term has been discussed by Mackie et al. (1976).

For further characterization of the two types, animals treated with toxic analogues of transmitter amines were investigated. In Type II terminals of planarians fixed immediately after the 30 min treatment with 5,6-DHT, all vesicles were dense (Fig. 3A). Single neurites were similarly labelled with

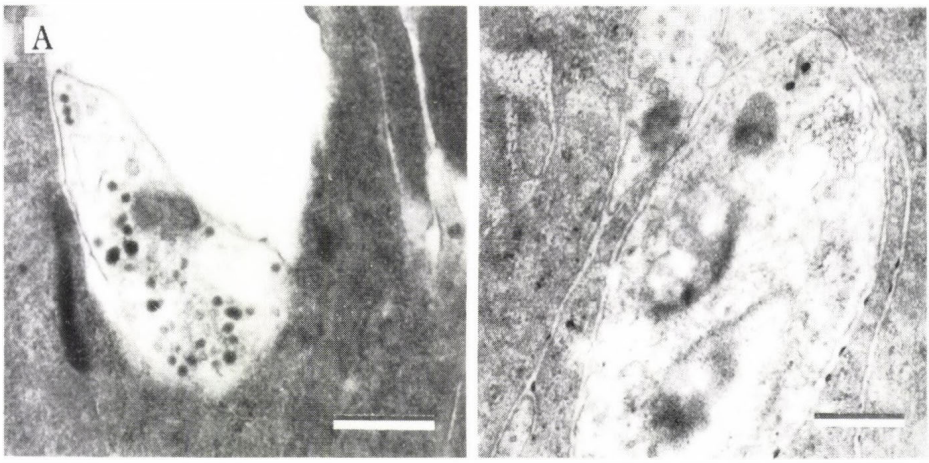

Fig. 3. Intraepithelial (Type II) axon terminals in planarians fixed immediately after treatment with 5,6-DHT (A) and one hour later (B). Scale 0.5 μm.

the incorporated toxin in deeper parts of the body, in particular in ventral nerve cords. However, no density was seen in Type I terminals. Long-term effects of 5,6-DHT on Type II terminals were manifested by progressive destructive changes (Fig. 3B). Treatment with 6-OHDA did not specifically affect either Type I or Type II nerve terminals. Selected deeper neurones were, however, strongly damaged by this toxin. Ultrastructural manifestations of degeneration could be seen in both the cell bodies and processes of these neurones. Such processes were found mainly in the muscular part of the ventral body wall where they made contacts with muscle cells.

DISCUSSION

Several lines of evidence indicate that in Dendrocoelum there exists a direct 5-HTergic innervation of the ventral epithelium, where 5-HT controls the activity of the ciliated cells.

First, immunocytochemistry provides evidence that the axon terminals, which enter the ventral epithelium and contact ciliated cells, contain 5-HT. Our findings thus confirm and ex-

tend previous data obtained by the histofluorescence method (Welsh and Williams 1970). Due to immunostaining, a more detailed picture of the distribution of 5-HT in planarians is now available. Among new findings is the fact that the ventral surface of the head, which does not participate in gliding, is not provided with a 5-HTergic nervous plexus and has an epithelium which is not supplied with 5-HTergic axons.

Second, the ability of intraepithelial nerve terminals to incorporate an exogenous indole was demonstrated by electron microscopy in animals treated with toxic analogues of transmitter monoamines.

Third, behavioural experiments show that the gliding activity is positively correlated with the availability of 5-HTP, i.e. with the rate of 5-HT synthesis. Since the ciliary locomotion was continuous in these experiments, it seems reasonable to assume that the gliding activity depended on the efficacy of the ciliary beat. In other words, our results suggest that the efficacy of the ciliary beat correlates positively with the rate of synthesis of 5-HT.

There is little doubt that all cilia are fundamentally automatic in their movement. They may, however, differ in their dependence on external factors. Gray (1928) classified the ciliary movement into three basic types: (a) cilia which are normally in an active state of movement independent of any external stimulus, (b) cilia which are motionless except when a stimulus is applied, and (c) cilia which are active but can be brought to rest by some type of inhibitory control. Locomotor cilia of D. lacteum seem to fall to Gray's Type b, since endogenous 5-HT appears to activate them in a concentration-dependent manner while reserpine stops their beating (Sakharov et al. 1986). The resting state may thus result from cessation of 5-HT secretion, rather than from active inhibition. This does not exclude the possibility that planarian locomotor cilia may be provided with a mechanism for emergent nervous inhibition, and may thus combine Gray's Type b and Type c. Our electron microscopic evidence seems to call for the dual innervation of the ventral epithelium.

To return to the question formulated at the beginning of the paper, our investigations have shown that in a flatworm the locomotor behaviour associated with hunting and feeding is activated by the same transmitter mechanism which is responsible for its activation in divergent advanced metazoans. The function of 5-HT in Dendrocoelum is not evidently restricted to an effect on ciliary beat. The list of 5-HT effects appears to include also relaxation of body muscles, activation of mucus secretion, inhibition of escape behaviour, and enhancement of feeding activity. All these effects are complementary, they characterize a specific behavioural state. 5-HT integrates behaviour of flatworms in a specific way, and its transmitter function is one mechanism for the realization of its integrative function. The integrative action of a transmitter substance on many target cells is probably the principle that is conserved from the earliest nervous systems.

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PEPTIDERGIC MODULATION OF NEUROTRANSMISSION IN THE MOLLUSCAN HEART

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When studying the regulation of the cardiac muscle sensitivity to acetylcholine (ACh), we put forward the suggestion about the involvement of neurohormones in this process. This suggestion is consistent with the observation that in the bivalve molluscs studied the highest heart sensitivity to ACh was strictly timed to spawning, while the lowest sensitivity was found during wintering (Nistratova et al., 1978; Nistratova, 1979, 1980). In October the ventricle of the freshwater bivalve *Anodonta* responds with a weak tonic reaction even to 10^{-3} M ACh, whereas in February, during the shedding of glochidia (larvae) from the gills, and in August, during the oviposition into the gills, the threshold to ACh is less than 10^{-7} M. These fluctuations are still greater in marine bivalves (*Spisula, Mactra, Mya* etc.), in which the thresholds to ACh during spawning are between 10^{-17} and 10^{-16} (the highest heart sensitivity to ACh was about one million times the lowest).

At present it is known that in molluscs neurosecretory substances are responsible for reproduction (Joosse, 1979; Motavkin, Varaksin, 1983). If our suggestion about the involvement of neurohormones in the regulation of the heart sensitivity to ACh is correct, there should be a correlation between the neurosecretory material (NSM) content of the parietovisceral (PVG) and cerebropleural ganglia (CPG), the heart sensitivity to ACh and the gonad maturation. Such a study was carried out together with Dr. A.A.Varaksin on the marine bivalve *Spisula*. In February, at the stage of rela-

tive rest, the number of Homori-positive cells in the ganglia was small. The NSM was granulated and located perinuclearly. The threshold to ACh was about 10^{-10} M. The ovary contained, predominantly, detritus and a few oogonia. In

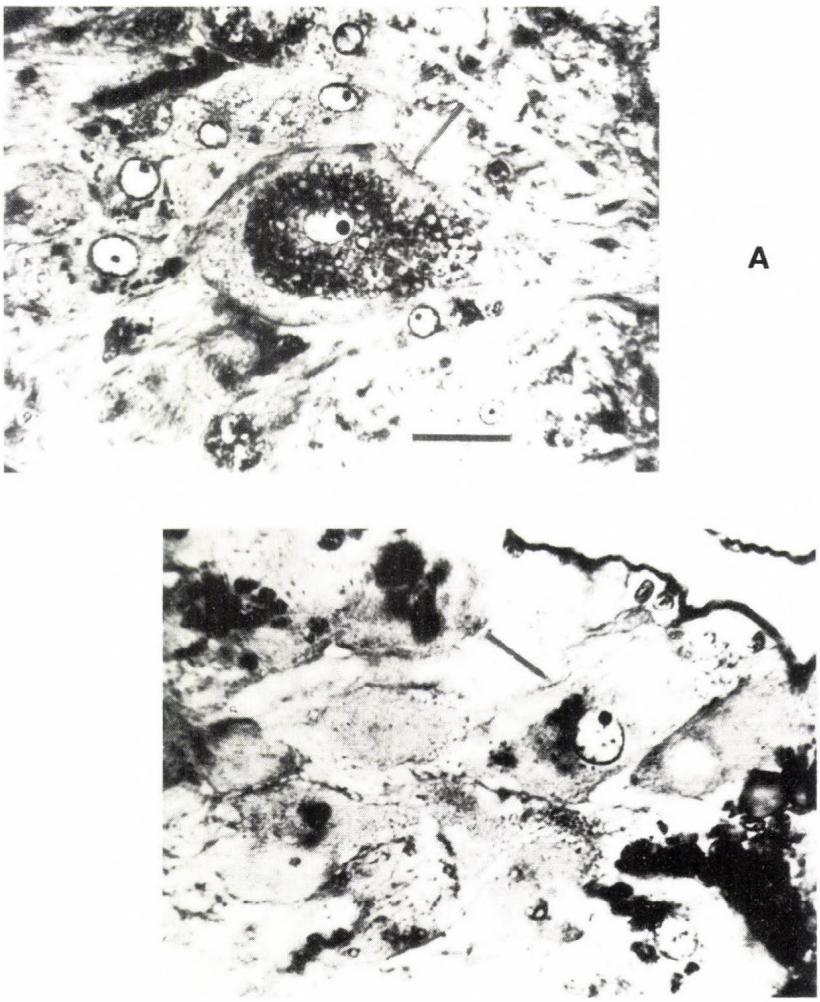

Fig. 1. Neurosecretory cells of the parietovisceral ganglion of *Spisula sachalinensis* a few days before (A) and just after spawning (B). Arrows indicate the cells with heavily stained granules. Paraldehyde fuchsin, poststaining with azan after Heidenhein. Scale bar, 100 µm.

July, before spawning, the ganglionic secretory cells were filled with the NSM (Fig. 1A) but the threshold to ACh was only slightly higher than in winter (10^{-12} - 10^{-11} M). The NSM is released from the ganglia only a few days before spawning. By this time the picture became different: the granules were lighter, vacuoles of various sizes appeared and neurosecretory cells soon became almost empty (Fig. 1B). It is at this time that the heart sensitivity to ACh sharply increased: the thresholds to ACh were between 10^{-17} and 10^{-16} M. This high sensitivity was preserved for some time after spawning and then returned to the initial, "winter" level.

There are at least two ways of the NSM transport to the target cells: (1) transport along the axon and direct action on the receptors after release from the nerve ending (Fahrmann, 1961; Baranyi-Barek, 1964; Motavkin, Varaksin, 1983) and (2) release from the nerve cell into the glia and, further, into hemolymph (Stuart et al., 1980; Rothman et al., 1983).

If there is an axonal flow, the NSM should be present in the molluscan heart. Electron microscopy studies (Drozdov, Nistratova, 1984) showed that in the nerve endings of the *Crenomytilus grayanus* ventricle there are at least two populations of peptidergic vesicles: large oval vesicles with a transverse diameter of 100-120 nm and small round ones, about 20 nm (Fig. 2). As a rule, the nerve endings with these populations of vesicles were located side by side and the large peptidergic vesicles occurred in the cytoplasm of the cardiac muscle cells as well. A similar picture was observed in the heart of gastropods (Nisbet, Plummer, 1966; Cottrell, Osborne, 1969).

The morphological data on the presence of NSM in the myocardium and its release from the ganglia during spawning do not, thus, contradict the possible involvement of these substances in the regulation of the heart sensitivity to ACh. This suggestion can also be tested by treating the myocardium with the neurosecretory substances or the extracts from the ganglia which contain these substances.

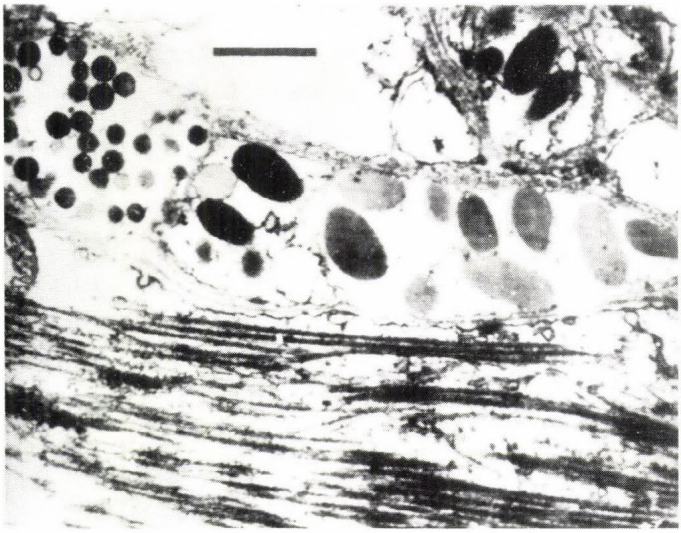

Fig. 2. Electron micrograph of nerve endings packed with dense-cored vesicles of different sizes in the ventricle of *Crenomytilus grayanus*. Scale bar, 200 nm.

Such a study was carried out on the heart of *Spisula* and *Anodonta*. The PVG or CPG aqueous extracts were added to the isolated ventricle mounted on a cannula. The extract itself had a powerful excitatory action: the diastolic tone was elevated and the force and rate of beating were enhanced even when the extract was diluted 100 times and more. Stable sensibilization to ACh could be seen only after washout. But these results require a lot of control experiments, since the neurosecretory cells constitute only a small part of the ganglion cell population and there are many other physiologically active substances in the extract. Our pharmacological analysis and the published data have shown that the extract contains ACh, 5-HT, FMRF-amide and some other substances.

The direct evidence of the involvement of the spawning-related NSM in the modulation of cholinergic transmission was obtained on the gastropod *Aplysia* which possesses the simplest endocrine system. In this mollusc, at the base of

pleurovisceral connectives the neurosecretory cells form two clusters, 200 to 400 neurons each (Frazier et al., 1967). These bag cells (Bc) are morphologically uniform and can easily be isolated, while in the other, even closely related species (e.g., in *Bursatella*) the neurosecretory cells are scattered all over the visceral ganglion and the ganglion itself is a part of CNS. The Bc contain electron-dense granules, 150 to 250 nm in size, and their endings are scattered in the connective tissue of neurohemal space.

Kupfermann (1967) was the first to show that the Bc aqueous extract induced egg laying when injected into another mollusc. The same effect was provoked by short-term excitation of the Bc (Ifshin et al., 1975). The biochemical and some physiological properties of the egg laying hormone (ELH) are now fairly well known. This alkaline neuropeptide with pI 9.0-9.3 and M_R 4835 Da (Arch et al., 1976) consists of 36 amino acids (Chiu et al.,1979). It is released into the neurohemal space and in the circulatory system and acts as a hormone. It dominates in the Bc extract while the other peptides (acidic peptide and bag cell peptides - BCP) exert no effect on spawning.

In this respect it was of interest whether the Bc extract would increase the heart sensitivity to ACh, thus reproducing the picture observed in nature during spawning. These studies were carried out on *Aplysia* and *Bursatella* (Nistratova et al., 1985).

There were two types of response of the *Aplysia* heart to ACh (Fig. 3):(1) decrease in the rate and the force of beating with a diastolic arrest (K_{ACh} is about 5.10^{-10} M) and (2) increase in tone with a systolic arrest (K_{ACh} is about 5.10^{-6} M). This appears to be due to the existence of two types of ACh receptors described for the other molluscan species: H type opening Cl^- channels and D type opening cationic channels (Elliott, 1980). It is rather difficult to obtain stable rhythmic activity for a long time: the *Aplysia* heart starts to beat irregularly and stops at last. At the same time the capacity for contractions and the excitatory action of ACh remain unchanged. That is why we employed

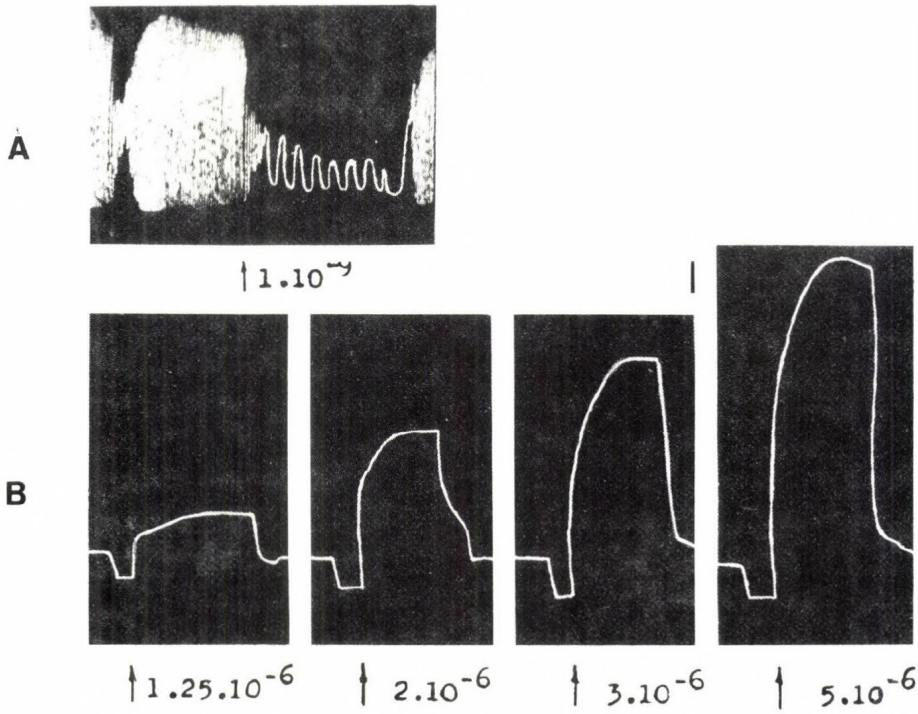

Fig. 3. Effects of ACh on the isolated *Aplysia* ventricle. A. Spontaneously beating heart. B. Quiescent heart. Arrows indicate addition of Ach, between the additions the ventricle is washed with sea water for 20 to 30 min to avoid desensitization. Numerals correspond to ACh molar concentrations.

mostly the quiescent heart as an assay for the Bc extract. Responses of the two receptors to the long-term ACh action and high ACH concentrations were also different: desensitization was much better expressed in the case of tonic receptor.

Direct effect of the Bc extract on the cardiac muscle. The Bc extract exerted a very pronounced effect on the isolated *Aplysia* ventricle: it restored the rhythmic activity in quiescent preparations and markedly potentiated the tone,

force and rate of beating. When diluting the extract (two Bc clusters in one ml of sea water) 2, 4, 10, and 100 times, the tone was the first to decrease, then the rate was reduced, while the force of beating was the last to be affected. The extract exerted a similar effect on the heart of *Bursatella* and *Strombus* and of the land gastropod *Zachrysia*. When the extract was kept for a few days at 5-10°, the "tonic" effect fully disappeared and the heart stimulation only remained. Benzoquinone (1.10^{-4} M) not only blocked the ACh effect, but also markedly reduced the tonic effect of the extract.

The PVG extract (without Bc) and the extract from the CNS of *Aplysia* induced the cardiac muscle stimulation, this latter being sometimes even more pronounced.

Modulating effect of the Bc extract on cholinergic transmission. The Bc extract enhanced the heart sensitivity to ACh. When applied to the ventricle for 2-3 min, the Bc extract markedly increased both the tone and the time of onset of regular beating (we determined the half-time, T_{50}). As can be seen in Fig. 4, the Bc extract increased the tonic response 4 times and T_{50} about 1.5. The cumulative effect is well expressed: the longer its action on the heart, the higher sensibilization to ACh. The stimulatory and sensibilizing effects of the Bc extract are probably not causally related; the strongly diluted extract (1:100 to 1:1000) caused no stimulation but enhanced the sensitivity to ACh. It is most likely that the force of heart beating and the heart sensitivity to ACh are enhanced by different substances present in the Bc extract. We have shown only that the heart is not stimulated by 5-HT, since its antagonist methysergide did not block the stimulation. In addition, 5-HT, unlike the Bc extract, was able to restore the beating of a quiescent heart.

The capacity of the Bc extract to relieve or markedly reduce desensitization to ACh, which arises in the heart of *Aplysia*, as well as of the other molluscs, after the repeated ACh action, is of especial interest. Usually, the

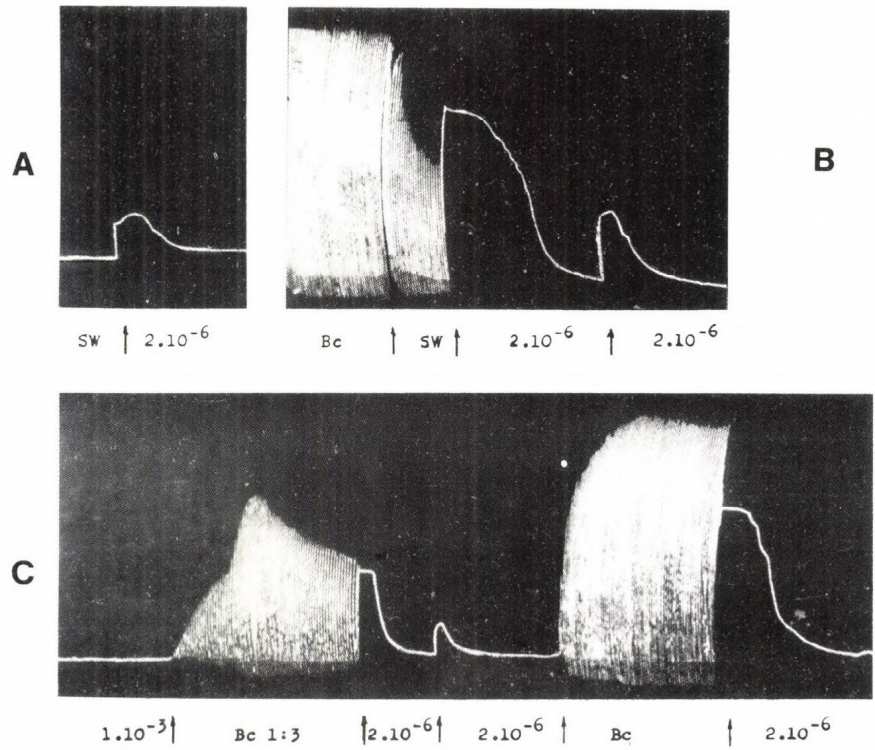

Fig. 4. The modulating effect of the Bc extract on the isolated *Aplysia* ventricle. A. Tonic reaction of the quiescent heart to ACh (2.10^{-6} M). B. The Bc extract induces spontaneous heart beating with a very high amplitude. The ACh effect is also enhanced. C. ACh (10^{-4}-10^{-3} M, 10 min) induces desensitization that can be relieved by the Bc extract. Bc, water-soluble extract. Arrows indicate the change of solutions. Numerals correspond to ACh molar concentrations.

desensitization induced by very high ACh concentrations (10^{-5} - 10^{-4} M) was relieved only after washout for 40 to 60 min. But if the Bc extract was applied for 1-2 min to the ventricle which did not respond to even 10^{-3} M ACh, the tonic reaction to ACh was immediately recovered (Fig. 4C). The higher the extract concentration, the more pronounced

its effect. The PVG extract slightly enhanced the tonic reaction but did not relieve desensitization, while the extract from the *Aplysia* CNS even blocked the ACh effect.

Hence, these results suggest that the Bc neuropeptides are able to modulate cholinergic transmission and both enhance the ACh response and relieve desensitization to ACh. Unfortunately, we used the Bc extract which contains not only ELH but also some other active polypeptides, including alpha-BCP, small cardiac peptides (SCP), etc. It is most likely that ELH exerts the modulating effect because in our experiments the stimulatory and sensibilizing effects were separated and the SCP could not be the active factor. Alpha-BCP is also an unlikely candidate since it is rapidly inactivated after the release and acts locally (Rittenhouse, Price, 1980, Sigvardt et al., 1986), unlike the ELH which is released into the circulatory system in sufficient amounts to affect the other tissues, including the gonads (Stuart et al., 1980; Rothman et al., 1983; Mayeri et al., 1985). As a result, profound behavioural changes occur: cessation of locomotion, onset of head-weaving movements and inhibition of feeding (Strumwasser et al., 1980).

The observed changes in the heart activity were also induced by the hormone circulating in hemolymph which both exerted the direct effect on the cardiac muscle and modified neurotransmission. The Bc extract potentiated the rate and amplitude of heart beating in *Aplysia* and induced rhythmic activity in quiescent preparations, these effects being long-term and reproducible on the hearts of the other gastropods, *Bursatella*, *Strombus*, and *Zachrysia*. Similar data were obtained by the other authors who showed that ELH increased the rate of heart beating and enhanced the burst-like firing of the neurons which regulate the heart activity (Smock et al., 1978).

By the capacity of ELH to modulate the myocardium cholinergic reaction seems to us the most interesting. Some biochemical and physiological data have been recently published which suggest the involvement of neuropeptides (especially, hormones of reproduction) in the regulation of

nervous processes. Jan et al. (1980) showed that LHRH-like hormones might mediate the late slow excitatory postsynaptic potential in the frog sympathetic ganglia, both LHRH and muscarinic agonists affecting the same K^+ channels (Adams, Brown, 1980). Corticosteroids modulate the reaction of the muscarinic ACh receptor in the membranes of the rat heart (Jacobsson et al., 1983). Lloyd (1986) described the postsynaptic modulating effect of SCP on the accessory radula closer which is innervated by the cholinergic motor neurons. FMRF-amide was shown to be involved in neurotransmission in *Helix* (Cottrell et al., 1983).

The possibility of modulating action of peptides is corroborated by the morphological data suggesting the coexistence of neuropeptides and classical transmitters. Besides ACh, various polypeptides were found in the cholinergic fibers: somatostatin in the *Bufo* atria (Campbell et al., 1982), vasoactive intestinal polypeptide in the cat neurons (Lundberg et al., 1979), LHRH in n.vagi innervating the frog heart (Jan et al., 1980). The joint release of the transmitter and the neuropeptide could enhance (or reduce) the basic effect.

When considering possible causes of changes in the heart sensitivity to the transmitter, the following might be taken into account: (1) intensification of the transmitter synthesis, (2) influence on the systems responsible for the transmitter destruction, (3) non-specific changes in the membrane conductance and electrical properties, and (4) changes in the interaction with the receptor itself. In our case one of these causes may be excluded, since in the ventricle of bivalve and gastropod molluscs ACh esterase, although present in small quantities, does not take part in cessation of the cholinergic reaction (Nistratova, 1980). All other possibilities may be tested by electrophysiological and biochemical methods. Nevertheless it is likely that ELH (or some other peptide) interacts with the ACh receptor because benzoquinone blocks both the ACh effect and the sensibilizing effect of the Bc extract.

It is now difficult to say whether the high sensitivity to ACh during spawning has any biological sense or just reflects the general rearrangement of the organism during this period. It is, however, clear that the release of ELH from the Bc into the circulatory system leads not only to sensibilization of the target cells to ACh, but also to the maintenance of the high level of sensitivity by relieving desensitization to the transmitter.

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DISCUSSION

CARLBERG, M.: Is it known whether extract from the bag cells prevents desensitization of ACh-receptors even in other tissue, e.g. mammalian neuromuscular junction?

NISTRATOVA, S.N.: As I know nobody made such an experiment but I think that Aplysia ELH will not modulate neurotransmission in mammals. The matter is that there are differences in hormones of oviposition in different animals. ELHs are similar enough

between species in the same genus of molluscs but in different subfamilies, orders and subclasses cross-injection of ELH failed to cause oviposition (Ram et al., 1977). In our experiments BC extract enhanced the ACh effect of Aplysia ventricle but blocked the ACh action upon the heart of Strombus and Bursatella. It might depend on M- or D-specificity of neurosecretory substances that induced egg-laying or maybe on differences in structure of ACh receptors (M^- or D^- types).

LUKOWIAK, K.: We have managed to keep Aplysia californica hearts going for hours. It is important to maintain them at the temperature which they are maintained in the aquarium. It is also extremely important that the proper perfusion pressure be maintained. When done properly, these hearts are extremely sensitive to neuropeptide modulation. For example Cawthorpe and others (1986) showed that in some preparations SCP_B at 10^{-17} M had both ionotrophic and chronotrophic effects which were reversed upon washout.

NISTRATOVA, S.N.: We worked with cannulated ventricle of Aplysia and it was not easy to maintain rhythmical activity for a long time. We tried to improve it at different temperature or pressure but without any success. Now we change the mode of preparation, the ligature being tied on a part of the aorta, or carry out experiments with electrically driven heart.

WALKER, R.J.: Did pretreatment with the peptide extract change the sensitivity of ventricle muscles to any other transmitters?

NISTRATOVA, S.N.: We should like to check this possibility on the Aplysia heart. Earlier we got some results with the effects of another polypeptide-substance P on the rat ileum. SP not only enhanced the acetylcholine effects but changed the ileum sensitivity to 5-HT, ATP and histamine.

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CELLULAR SUBSTRATES OF ASSOCIATIVE MEMORY IN MOLLUSK AND MAMMAL

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Reflection on our own memories suggests that they are always complex. They never involve one bit of information in isolation of other bits. Rather, they consist of constellations of bits and the relationships of those bits in time and space.

A remembered image of a face is a total effect of the relation in space of its parts: nose, mouth, eyes, etc. This apparent universal complexity of our memories suggests the possibility of a unifying simplicity: what we remember are really the relationships between stimuli rather than the stimuli themselves, and any given memory represents a particular collection or set of relationships. This relational quality of human memory is more familiar to us as its associative nature.

Pavlovian conditioning is one particular form of associate learning which is extremely sensitive to the time interval between the associated stimuli. This dependence of conditioning on the time interval between stimuli provides a constraint which should also be reflected by the cellular physiology which underlies the behavior. It allows us to distinguish between cellular changes due to sensory and motor experience in general vs. learning.

What I would like to describe are a series of parallels we have observed between Pavlovian conditioning of the snail and the rabbit. These parallels were apparent at several levels of biologic complexity: behavior, neuronal excitability and membrane channels, and the molecular regulation of these membrane channels.

Behavioral Parallels

During Pavlovian conditioning of both the snail Hermissenda and the rabbit, a predictive relationship between two discrete stimuli is learned. As a result of conditioning the snail Hermissenda, a light stimulus elicits "clinging" which closely resembles the effect of rotation. As a result of conditioning the rabbit, a tone elicits an eyeblink similar to that elicited by corneal touch. This transfer of a behavioral response from one stimulus to a second associated stimulus is one of the clearest indices that an association between stimuli has been learned. Many other characteristics, however, common to both Hermissenda and rabbit conditioning, contribute to a definitive demonstration of Pavlovian conditioning in these two species. These include precise temporal and stimulus specificity, acquisition, extinction, a requirement for contingency, savings, and duration for weeks or longer. There is also no long-term sensitization (a non-associate arousal-like effect) for either the snail or rabbit learning. Thus, the underlying cellular mechanisms for the Pavlovian conditioning of these species cannot be obscured by or confused with underlying mechanisms for non-associative behavior modification such as sensitization.

Biophysical Parallels

For both the snail and the rabbit, cellular transformations accompany transformations of behavior of the intact living animals. In addition to correlation of cellular and behavioral change, however, satisfaction of other criteria helps identify cellular mechanisms as an actual means of memory storage. To store the memory, the cellular changes must be localized, i.e. shown to be intrinsic to specific cellular loci - thus they should persist at these sites even after the sites have been isolated from all other neural elements and modulation. To store the memory, the cellular changes must also predict changes of signal flow through neuronal pathways and out of the nervous system in a way which causes some or all of the learned behavioral change. And final confirmation of this predictive capacity should be

demonstrated by producing the learned cellular changes at these sites in intact animals and thereby actually producing the learned behavior.

By these criteria, changes within specific Hermissenda neurons, the Type B cells, were, in fact, storing the learned association. The Type B cells are a locus of convergence between the visual and vestibular pathways. K^+ current reduction across the Type B soma membrane had a duration (at least for days) not previously observed for ionic currents across membranes of differentiated neurons. This conditioning-specific K^+ current reduction represents biophysics within a new temporal domain - a domain of days rather than msecs and seconds.

More recently, John Disterhoft, Doug Coulter and I have obtained evidence of the same long-lasting transformation of specific voltage-dependent K^+ currents in hippocampal slices isolated on days after conditioning of the nictitating membrane response of the rabbit.

In Hermissenda, information from distinct sensory stimuli comes together, i.e. converges on sensory receptors in addition to second order neurons and central interneurons. In vertebrate brains, such as that of the rabbit, convergence occurs more typically much further into the nervous system, i.e. more centrally. But there is a great deal of evidence that the biophysical properties are quite similar for loci of convergence in Hermissenda and mammals. It was not entirely surprising, therefore, that a biophysical memory record we found in rabbits days after conditioning was quite similar to that we found in Hermissenda.

Although it has not yet proven possible to trace the flow of sensory information through the hippocampus during conditioning of the rabbit in a step-by-step manner as was possible for the snail Hermissenda, there are a number of experiments which implicate the hippocampus as an essential structure for storing certain types of information about the Pavlovian conditioning experience. Lesions of the hippocampus do not abolish the eyeblink response but they do impair retention: they greatly enhance extinction of the conditioned response. These lesions also enhance stimulus

generalization and disrupt discrimination reversal, blocking, sensory preconditioning and latent inhibition.

Biochemical Parallels

Having considered behavioral and biophysical parallels, extension of our analysis of Pavlovian conditioning to the molecular level has revealed further parallels between storage mechanisms in the snail Hermissenda and the rabbit.

Let us first review the sequence of cellular and molecular events as we understand it thus far for Hermissenda.

The visual-vestibular network (including pharmacologic effects on membrane currents) responds with increased and prolonged depolarization of the Type B cell to light precisely paired with rotation than to unpaired stimuli or to either simulus alone. In response to repeated light-rotation pairings, as occurs during training, depolarization of the Type B cell becomes progressively larger and more prolonged, i.e. it accumulates. Accompanying this depolarization is prolonged activation of voltage-dependent influx of Ca^{++} as well as periodic marked elevation of Ca_i^{++}. On days after training, there is no depolarization of the Type B cell, no prolonged activation of Ca^{++} influx and no Ca^{++} elevation. Yet the K^+ currents activated by CS presentations are unequivocally reduced for the conditioned group, suggesting that conditions such as Ca_i^{++} elevation have left a trace.

On a short time scale, Ca^{++} iontophoresis under voltage-clamp reduces K^+ currents. On a larger time scale, the molecular basis for this stored K^+ current reduction, which is recalled by depolarization of the Type B cell membrane, appears at least, in part, to be due to persistent difference of a Ca^{++} and lipid-activated-kinase-mediated phosphorylation. Conditioning-specific differences in phosphorylation of a 20,000 MW protein were, in fact, measured in isolated Hermissenda eyes hours after training. This 20,000 MW protein was shown with in vivo and in vitro assays by Joseph Neary and Shigetaka Naitoh in our laboratory to be a substrate for two

phosphorylating enzymes: C-kinase as well as Ca^{++}/calmodulin-type II kinase. Injection of either of these two enzymes together with Ca^{++}-loading conditions precisely simulates the effects of conditioning on the two K^+ currents, I_A and $I_{Ca^{++}\text{-}K^+}$. Blockers acting on the Ca^{++}/calmodulin dependent and C-kinase endogenous to the Type B cell have the expected opposite effects on the K^+ currents. Furthermore, pharmacologic activation of the C-kinase (e.g. with phorbol ester), together with Ca^{++} loading, also simulates the effects of conditioning on the K^+ currents. Finally, prolonged depolarization of Hermissenda neurons, i.e. exposure to conditions which simulate electrophysiologic effects of conditioning modifies phosphorylation of the 20,000 MW as well as 25,000 MW proteins. These and other experiments (such as injection of inositol trisphosphate) suggest that light-rotation pairings, via Ca_i^{++} elevation and elevation of DG, activate both the C-kinase and the Ca^{++}/CAM-dependent kinase to alter phosphorylation of proteins that either regulate or are a part of K^+ channels within neuronal membranes. Altered phosphorylation, perhaps via movement of C-kinase from the cytoplasm into the membrane compartment, which activates the C-kinase and reduces K^+ currents, occurs within a time domain of at least many hours and possibly days. For time domains extending at least many days and possibly longer, Hermissenda Pavlovian conditioning involves modification of protein synthesis which may arise, in part, as a consequence of C-kinase activation. Dr. Tom Nelson has recently shown that mRNA turnover in the same isolated eyes for which the phosphorylation differences were measured dramatically increases 36 hours and persists even 4 days after the conditioning has been completed. For the conditioned group, there is an unequivocal correlation between the extent of the learning, i.e. the magnitude of the learned response and the magnitude of the increase in mRNA turnover. Thus, just as was possible for the conditioning-specific change of K^+ currents, it was possible to relate the learned behavioral change of intact animals to learning-induced molecular changes at the cellular level.

For Hermissenda, the cellular sequence during memory storage extends from conditioning-specific release of second messengers, to persistent modification of protein phosphorylation, to long-lasting alterations of protein synthesis. C-kinase activation may not only affect the phosphorylation differences, it may also be involved in at least initiating alteration of protein synthesis. This was suggested by the finding that pharmacologic activation of C-kinase causes marked differences in synthesis of Hermissenda neuronal proteins. Furthermore, inhibition of protein synthesis prolongs C-kinase effects on Hermissenda membrane currents.

Clear indication of C-kinase involvement in storing records of rabbit associations in the hippocampus has also recently emerged from the work of Barry Bank in our laboratory. At least 24 hours after classically conditioning a rabbit (or control procedures) regions of the brain were sliced and specific portions isolated from microgel analysis of cytoplasmic and membrane fractions. Rabbit nictitating membrane conditioning was found to cause a marked shift of C-kinase distribution: from the cytoplasm into the membrane compartment of the microdissected CA1 neuron region. Radioactive phorbol ester provided an autoradiographic means of localizing the conditioning-specific translocation to the proximal dendritic region of the CA1 neurons. Finally, just as for Hermissenda conditioning, pharmacologic activation of the C-kinase reproduced the conditioning-specific alteration of the K^+ current measured in the same CA1 neurons, and can account for their increased excitability.

The snail and rabbit results suggest a synthesis:

An animal begins to learn an association between stimuli when signals from the environment travel along neuronal pathways which converge. Appropriately timed signals interact first electrically at post-synaptic sites of convergence (Figure 1). Post-synaptic interaction leads to interaction of second messengers which activate Ca^{++}-dependent phosphorylating pathways. A persistent biophysical record of the signal interaction results in a biophysical record: persistent reduction of voltage-

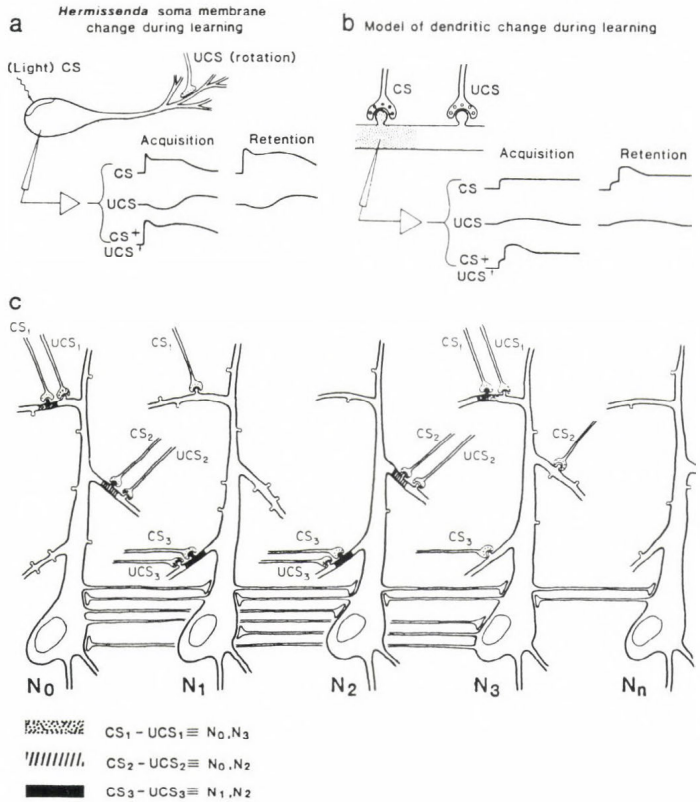

Fig. 1 (a) Schematic summary of conditioning-induced soma membrane changes in Hermissenda. Paired light and rotation are followed in the type B cell soma by an enhanced long-lasting depolarizing response (LLD) to light. Repeated pairings (lower left records) lead to cumulative membrane depolarization, enhanced response during and after the light, decrease voltage-dependent K^+ currents, increased input resistance, and thus increased excitability. One the days after training, that is, during retention of the learning, the cumulative depolarization is no longer present, whereas the other changes remain (24, 27, 30). (b) Model of conditioning-induced membrane changes in a dendritic branch. The genesis of these membrane changes could arise in a manner analogous to that observed for Hermissenda type B cells. (c) Hypothetical neural system changes during associative learning. Potential conditioned stimulus pathways synapse on postsynaptic dendritic membrane in proximity to postsynaptic sites for unconditioned stimulus pathways. Repetition of temporally associated stimulation of preexisting conditioned and unconditioned stimuli inputs which share a common postsynaptic dendritic branch cause persistent increases of postsynaptic excitability at the conditioned stimulus site (shaded areas). For a given set of neurons ($N_0 \ldots N_n$) different combinations of neurons will show increased excitability in response to a conditioned stimulus, depending on which stimuli were paired during training and depending on the genetically constrained features of the already formed neural systems. Horizontal processes ending on cell bodies represent inhibitory presynaptic endings.

dependent K^+ currents. Underlying the biophysical record in an intermediate time domain, one of days, is a persistent activation of C-kinase, via its translocation from the cytoplasmic into the membrane compartments. Extending into more permanent time domains, the biophysical record would depend on alterations of steps in protein synthesis such as the increased mRNA turnover found for Hermissenda.

What the snail's eye tells the rabbit's brain, then, and what has been heard at least by the rabbit hippocampus, is how stimulus relationships can be stored at a cellular level for later recall.

Even for the snail, however, the relationship between stimuli was encoded not by a single synaptic interaction but by a network of interactions within the visual-vestibular system of Hermissenda. What the snail's eye has not revealed is the wiring of the critical hippocampal networks or the integrative operations by the vast array of neurons within these networks during learning, storage and recall of the associative memory. Nevertheless, the kinds of molecular insights which appear to apply to vastly different species such as the snail and the rabbit should motivate the design of biochemical probes which could tag a distribution of neurons participating in the memory process. Such probes, by uncovering which cells show steady-state biochemical shifts with learning, then, could give new meaning to what has for so long been searched for by so many: i.e. traces of memory within the brain.

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DISCUSSION

BALABAN, P.M.: I have two questions: (1) Plastic changes that you have demonstrated last for several days. It is known that turnover of proteins is limited usually by 10-20 hours. How can the changes in the protein part of the channel persist for such a long time without involvement of a genome?

(2) It seems that the learning paradigm that you have used is not purely Pavlovian. In a Pavlovian paradigm it must be possible to elaborate several differing conditioned reactions to the same CS. In your case it is only one behavioural reaction-shortening. Have you tried to elaborate some other reaction?

ALKON, D.L.: 1. First, Dr. Thomas Nelson in our laboratory has recently demonstrated a marked increase of mRNA turnover in cells isolated from conditioned but not control animals 36 hours after conditioning. This increase, which could reflect involvement of the genome, is remarkably correlated with the degree of learning. Second, C-kinase translocation could confer relative invulnerability to cytoplasmic protease and thus preserve the C-kinase in its active form for an unusually long period.

2. We have demonstrated that when the CS, light, is paired with a UCS, rotation, with a specific vectorial restriction such that the animal's tail rather than its head points to the centre of rotation, light will subsequently elicit more rapid movement (and thus lengthening) toward the light source.

HONEGGER, H.W.: What reaction is catalysed when protein kinase C is incorporated into the membrane?

ALKON, D.L.: In Hermissenda we have measured a conditioning-specific difference in phosphorylation of a 20,000 MW protein. This protein is a substrate for both protein kinase C and Ca^{+}/calmodulin-dependent kinase.

Activation of the kinase C regulates phosphorylation at this and other low molecular weight proteins. We do not yet know

whether this protein is a part of or simply a regulator of K^+ channel(s).

JANSE, C.: Normally dogs do not respond with salivation to ringing a bell, they can, however, learn it. Does that mean that there are silent synapses which can be turned on?

ALKON, D.L.: I would not necessarily expect that there are truly silent synapses. Rather, I would expect that the efficacy of these synapses is insufficient to allow information flow triggered by the sound of the bell to elicit salivation. Conditioning, then, would enhance this efficacy to produce a conditioned response.

LUKOWIAK, K.: What happens to the conditioned response (eye blink in the rabbit) when you surgically remove the hippocampus?

ALKON, D.L.: Ablation at the hippocampus does not eliminate the conditioned or unconditioned responses but does clearly modify the rate at which the conditioned response is extinguished (as recently demonstrated by Dr. John Distertott at Northwestern University). This effect at hippocampal ablation is consistent with other experiments which implicate a hippocampal role in stimulus generalization, blocking and sensory preconditioning.

van der WILT, G.: In your model, what mechanisms could be responsible for the differential abilities of an animal in associative learning?

ALKON, D.L.: We have indeed encountered rather drastic differences in both Hermissenda and rabbit learning abilities. What features at the neural systems, be they biophysical and/or biochemical, account for such differences must be the subject of future studies.

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ROLE OF SEROTONERGIC CELLS IN AVERSIVE LEARNING IN HELIX

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INTRODUCTION

There is a growing body of experimental data implicating serotonin (5-HT) in a wide range of memory processes and conditioned reactions in molluscs (Kandel and Schwartz, 1982) as well as in vertebrates (Ögren, 1982). However, experimental evidence concerning the relationship between the level of 5-HT in the nervous system and associative learning is contradictory. Highly variable effects have been reported following depletion of central 5-HT content as well as after increase of 5-HT concentration. Nevertheless, the general conclusion made by the majority of investigators is that 5-HT participates in aversive learning (Ögren, 1982).

Investigation of the role of 5-HT in learning would necessitate, on the one hand, selective ablation of all 5-HT-ergic cells, and, on the other hand, selective stimulation of these cells. Recently several clusters of 5-HT-ergic cells were investigated in Helix (Balaban et al., 1985). Neurotoxins 5,6- and 5,7-dihydroxytryptamine (5,7-DHT) were reported to ablate selectively the terminals of serotonergic neurons in invertebrates (Hiripi et al., 1981; Glover and Kramer, 1982). These data encouraged us to investigate the role of serotonin in aversive learning in Helix. Aversive learning was demonstrated in this animal earlier (Maximova and Balaban, 1984).

METHODS

Conventional electrophysiological techniques for intracellular recording of identified neurons were used. Neurophysiological experiments were carried out in either a semi-intact preparation (Maximova and Balaban, 1984), a lip-central nervous system (CNS) preparation (Balaban et al., 1987), or an isolated CNS preparation. Suction electrodes were used for the electrical stimulation of nerves and local stimulation of ganglia.

In behavioural experiments the snail's shell was glued to a holder, while the foot of the animal was on a plastic ball floating in water. This experimental set-up allows the animal to move freely, while repeated tactile stimuli may be delivered to the same place of the skin. The amplitude of the behavioural responses was recorded by a photocell or using a video monitor. Tactile stimuli of different intensities were applied by means of calibrated von Frey hairs. Twenty mg per kg body weight of 5,7-DHT (SIGMA) dissolved in a saline solution with 0.1% ascorbic acid was injected into the haemocoelom of the snails 5-15 days prior to the experiments.

RESULTS

Effect of 5,7-DHT treatment on aversive learning in intact snails

Effect of decreased concentration of 5-HT in the CNS due to administration of neurotoxin 5,7-DHT was investigated in behavioural experiments. After 5 paired presentations of food and electrical stimulation, the number of feeding responses to food decreased from 90% to 15% in a group of control, intact snails (sham-injected). This decrease is characteristic of aversive learning in untreated animals. In both the experimental group, injected previously with 5,7-DHT, and the sham-injected pseudo-conditioned group the number of feeding responses did not change with pairing. These results suggest an essential role for serotonergic neural systems in aversive conditioning (for details see Balaban et al., 1987).

It is important to note that in 5,7-DHT-treated snails it is possible to obtain conditioned responses with food reinforcement, i.e., in neurotoxin-treated animals only conditioning to noxious stimuli was impaired. Injection of 5,7-DHT after the acquisition of an aversive conditioned response did not impair the expression of conditioned response. This suggests that 5-HT is necessary only at the stage of acquisition of the conditioned response.

Aversive learning in juvenile snails with low content of serotonin in the CNS

The presence and localization of biogenic amines were studied with the glyoxylic fluorescence histochemical technique (for details see Zakharov and Balaban, 1987). In the CNS of newborn animals 5-HT-containing neurons were not found. It was thought that the cells in newborn animals might be too small to be revealed by this technique, but in the CNS of 4-month-old snails the 5-HT-containing neurons were readily seen in cerebral and pedal ganglia, while the CNS size at this age is only slightly larger than in the newborns. Thus, in the postnatal period, the serotonin content is very low in the nervous system of the snail.

Using the same methods for conditioned reflex elaboration as were used for adult animals, the aversive conditioning paradigm was applied both to a group of newborn snails, and to a group of 4-5 months old snails. Before training sessions, all animals exhibited feeding responses in 90-100% of trials. After 9-20 pairings of the food with the noxious stimulus, hungry snails aged 4-5 months refused to take food in 90% of test trials, while snails younger than 1 month ate paired type of food in 90-100% of test trials even after 20-24 paired trials. Pseudo-conditioned snails of all ages exhibited feeding responses in 95-100% of the test trials. Thus, in the early stages of postnatal development the capacity to acquire aversively conditioned responses is absent in snails. The coincidence of these holes in the behavioural repertoire of juvenile snails and in 5,7-DHT-treated adult snails allows us to suggest that it is

the difference in 5-HT levels in juvenile, as compared to adult snails, that explains the absence of aversive conditioning.

Serotonin as a reinforcement

The presented data suggest the necessity of a certain level of 5-HT in the CNS for acquiring a novel aversive reaction. Using a next stage of reduction of experimental situation, we have tried to investigate the role of serotonin in a reduced preparation of CNS-chemoreceptors. Application of a drop of carrot juice containing chemoreceptors on the lip was used as a conditioned stimulus, while bath application of 5-HT (final concentration 10^{-5} M) for 2 min was used as a reinforcement.

It was shown earlier (Maximova and Balaban, 1984) that after 5-15 paired presentations of a drop of carrot juice on the lip in such preparation, and a strong pallial nerve stimulation, postsynaptic potentials and action potentials (which were absent prior to training) were recorded in response to juice in command neurons controlling avoidance behaviour. A novel spike response may be considered equivalent to the avoidance reaction of the animal, since action potentials in command neurons controlling this form of behaviour always trigger avoidance reactions (Balaban, 1983).

In the present series of experiments the conditioned stimulus (CS) was the same, but nerve stimulation was changed by bath application of 5-HT. In all experiments activity of giant metacerebral cells involved in the feeding behaviour of the snail was recorded, as well as activity of the command neurons for the avoidance behaviour.

Representative records are shown in Fig. 1. At the beginning of the experiment a drop of juice (10 tests) evoked in all cases a spike response in feeding behaviour neuron and no response in aversive behaviour neuron (Fig. 1A). Three explicitly unpaired presentations of 5-HT and juice (interstimulus interval 5-15 min) elicited only general increase of excitability, but no response to food in command neurons for avoidance behaviour (Fig. 1B). Forty minutes after the last of 3 paired presentations of juice and 5-HT a spike response to juice appears

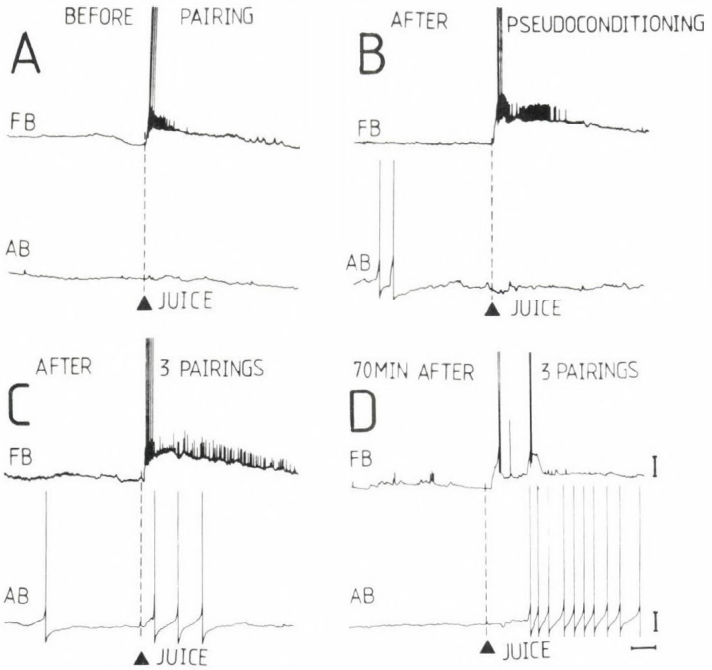

Fig. 1. Responses to juice application on the lip (triangle) in synchronously recorded right giant metacerebral cell (upper trace) and command neuron for avoidance behaviour at the beginning of the experiment (A), 40 min after 3 unpaired presentations of the juice and 5-HT (B), and 40 (C) and 70 (D) min after 3 presentations (paired) of juice and 5-HT.

in command neurons (Fig. 1C). This response usually increases later even in the background of decreased excitability (no spikes in Fig. 1D in spontaneous activity). Similar results were obtained in 7 out of 8 trained snails (25 neurons were recorded), while in all 8 pseudoconditioned animals (28 neurons) no long-lasting changes were seen.

These data allow to suggest that a certain amount of 5-Ht is not only necessary, but sufficient at least for some components of associative learning in experimental situation of acquired aversion in snail.

Effect of stimulation of 5-HT-ergic cells

Several groups of serotonergic cells are revealed in the CNS of snail by histochemical technique and by vital staining with oxidized 5,7-DHT (Balaban et al., 1985). It was found that extracellular stimulation of a group of 5-HT-ergic cells located in the rostral part of pedal ganglia affects the amplitude of synaptic potentials in command neurons for avoidance behaviour in the same way as application of 5-HT. Besides this effect, stimulation of the serotonergic cells elicits changes of excitability in command neurons which are not mimicked by 5-HT application, but are readily evoked by adequate stimulation.

We have studied these serotonergic cells using intracellular recording in the semi-intact preparation. It was found that serotonergic cells usually fire tonically, and increase frequency of firing in response to tactile stimulation (Fig. 2).

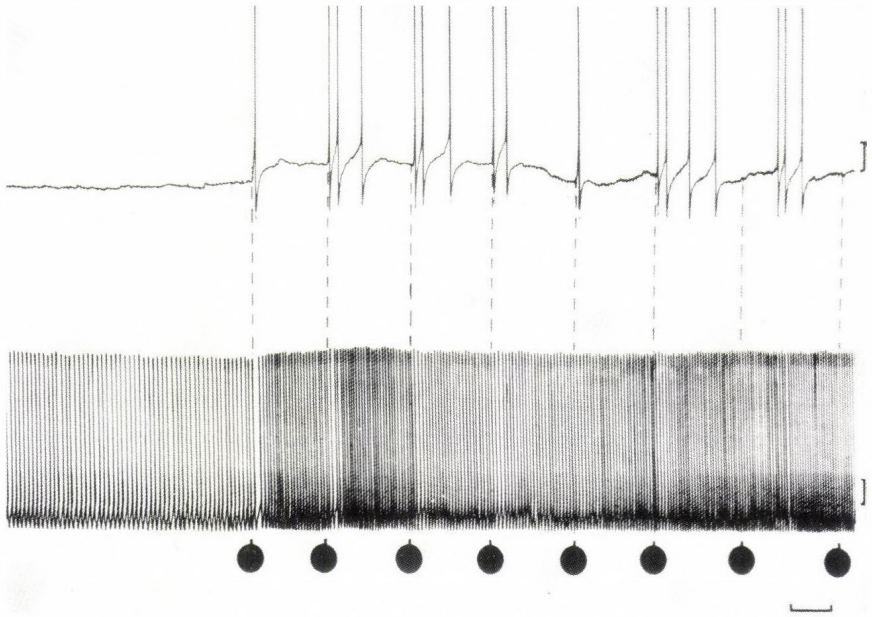

Fig. 2. Synchronously recorded responses of command neurons for avoidance behaviour (upper trace) and tonically active identified serotonergic pedal neuron (lower trace) to rhythmic tactile stimulation (filled dots) of the foot in the semi-intact preparation. Note coincidence of sensitization of spike response in command neurons and increase of firing frequency in 5-HT-ergic cell. Calibration 10 mV, 10 s.

This increase coincides with depolarizing wave and sensitization of spike response in command neurons for avoidance behaviour. In order to establish the origin of this wave, intracellular polarization of one cell was used. An example of a typical experiment is shown in Fig. 3. Release from hyperpolarization of serotonergic cell led to spiking in this cell and a depolarizing wave in command neuron. Increase of tonic firing frequency in 5-HT-ergic cell by means of depolarization elicited an increase of depolarizing wave and a pronounced increase of EPSPs amplitude in command neurons (Fig. 3). Hyperpolarization of 5-HT-ergic cell to the nonspiking level led to decrease of EPSPs amplitude in command neuron (Fig. 3).

The data suggest that identified group of 5-HT-ergic cells in pedal ganglia can influence the network underlying the avoidance behaviour in modulatory fashion. These neurons respond to adequate noxious stimuli, and, therefore, can constitute a part of reinforcement.

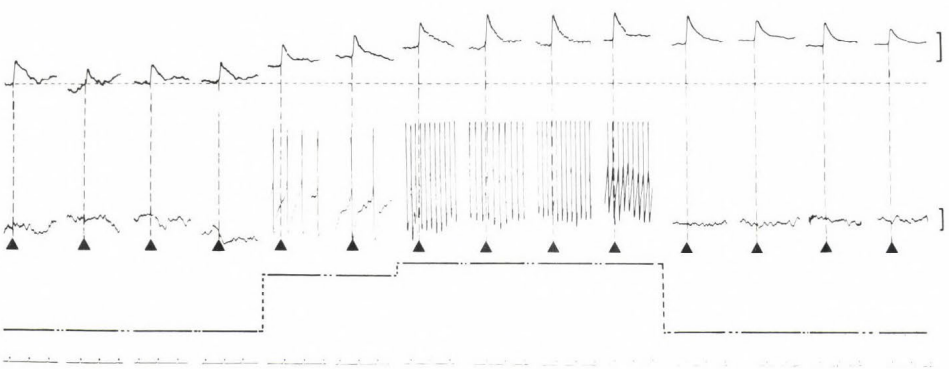

Fig. 3. Synchronously recorded responses of command neuron for avoidance behaviour (upper trace) and identified 5-HT-ergic cell (lower trace) to rhythmic (1 per 20 s) stimulation (triangles) of the cutaneous pedal nerves. Successive fragments of record are shown. 5-HT-ergic cell is hyperpolarized to prevent spiking (changes of polarizing current are shown by dotted line below the trace). Depolarization and spikes in 5-HT-ergic cells evoke increase of EPSPs amplitude and a depolarizing wave in command neurons. Calibration 10 mV, 1 s.

DISCUSSION

General description of neuronal net involved in aversive learning

A simplified schematic representation of interconnections of neurons involved in feeding and avoidance behaviour is shown in Fig. 4. It must be noted that in Helix CNS there exist several groups of 5-HT-ergic cells, but modulatory effect on aversive reactions is exerted only by 5-HT-ergic cells in pedal ganglia. These cells release 5-HT and some unknown factor evoking: (1) increase of excitability in command neurons; (2) increase

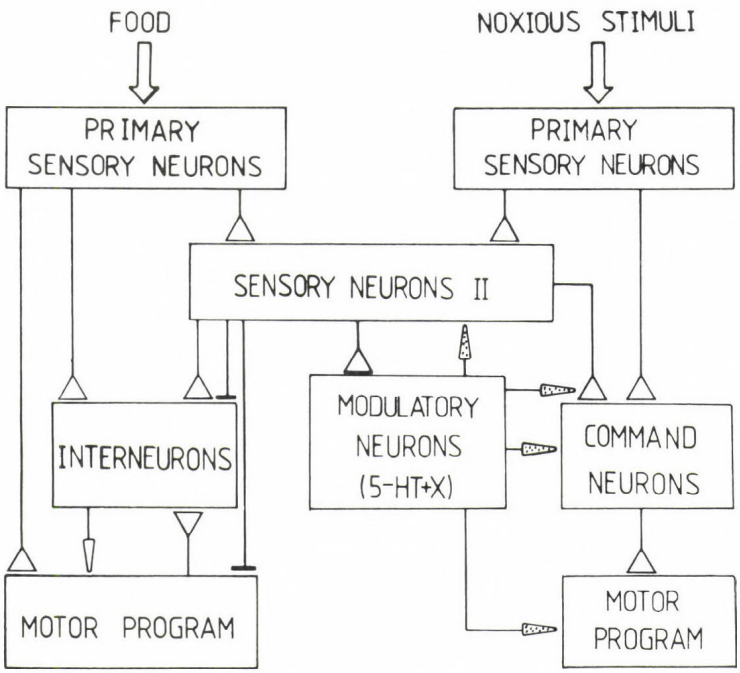

Fig. 4. Simplified schematic representation of neuronal net underlying feeding and avoidance behaviour. After pairings of food and noxious stimuli, presentation of food evoked spike reaction of command neurons and activation of motor program. Sensory neurons of the second order constitute a link between two forms of behaviour, and their synapses with the command neurons are the most possible sites of long-term changes. Triangles - excitatory connection, bars - inhibitory. Stippled arrows - modulatory excitatory influence.

of excitability and increase of spike duration in sensory neurons presynaptic to command neurons (Balaban, 1987); (3) increase of tonic activity in motor neurons (unpublished observation, Chistyakova). Due to these changes a previously under-threshold input evokes a spike reaction in the command neurons for the avoidance behaviour, and a corresponding behavioural reaction. As it is shown, modulatory neurons are activated tonically by phasic noxious stimuli which usually constitute a reinforcement.

Three loci in which conditioned stimuli may interact with unconditioned (noxious) ones can be outlined: (1) sensory neurons of the second order, (2) their terminals at the processes of command neurons, and (3) the command neurons for avoidance behaviour. The changes are noted in all these points, but it is not known how they are related to the long-term memory.

Short-term versus long-term memory

There is no problem today with mechanisms of a short-term memory. Changes in neuronal excitability evoked by adequate stimulation last minutes and tenths of minutes. Changes in spike duration in sensory neurons are responsible for sensitization and participate in conditioning (Carew, 1987).

But there exists one general limitation which does not allow to think that these changes in cells involved in a "direct pathway" for the conditioned and/or unconditioned reactions can underlie the long-term memory. This limitation is a simple one: the same sensory and motor neurons can be involved in functionally different behavioural situations. It means that if these changes are long-term ones, the whole behaviour must change after elaboration of 1 conditioned reflex. But when nonspecific sensitization disappears, the behavioural performance is quite normal in all respects except the response to the paired stimulus. We consider that long-term changes are not likely to occur in neurons involved in a direct pathway from stimulus to reaction.

A speculative alternative can be proposed. A special class of neurons is responsible for acquisition of a long-term me-

mory. It is the command neurons whose function is to detect stimuli with specific properties (noxious stimuli in the case of aversive learning) and to trigger a specific reaction. In such a conceptual framework aversive learning is only an identification of a specific type of food as noxious stimulus based on previous coincidence in time during pairing. The behaviour of the animal remains undisturbed in this case except for the response to a conditioned stimulus. This suggestion does not deny any findings of mechanisms of plasticity described in snails, but transfers these mechanisms to a specific class of neurons.

Is 5-HT a specific transmitter for reinforcement?

The obvious answer is "no" in general, and "yes" for aversive learning in snail. At least part of reinforcement necessary for aversive learning in snails is mediated by 5-HT. It must be noted that even in such relatively simple animals as snails, 5-HT has additional functions: activation of locomotion, modulation of feeding. In higher animals the function of 5-HT is more complex and intrinsic, and it is difficult to analyse its role in behaviour.

We think that every behaviour has its own modulator: one of transmitters, preferably those with a durable action - biogenic amines, peptides. These modulators are necessary for acquisition of memory ("give permission"), because a special motivational and functional state is needed for learning, but do not take part is reproduction of the memory trace.

Role of 5-HT-ergic cells in aversion

It seems that 5-HT plays the same role in aversive learning in Helix as in conditioning in Aplysia (Carew, 1987). Spikes in sensory neurons (presynaptic for command neurons) increase their duration due to increased level of 5-HT. Consequently, synaptic input to command neurons increases (Balaban, 1987). Such increase is not seen in spikes of command neurons and motor neurons.

The data allow to suggest that 5-HT-ergic cells modulate avoidance behaviour of the snail. Activation of these neurons, and consequent increase of 5-HT levels, create a new functional state which is a necessary stage for acquisition of a new aversive reaction. 5-HT-ergic cells "give permission" for formation of a long-term memory.

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DISCUSSION

ARVANOV, V.: As far as I remember in an early paper of Prof. Sakharov and coworkers the seasonal dependence of serotonin sensitivity of RPa1 neuron was demonstrated. Have you found any dependence of learning processes on the season?

BALABAN, P.M.: Indeed, we have found seasonal dependence of aversive learning. In a period from February to May it is almost impossible to elaborate aversive conditioning. We suggest that it depends on serotonin level.

ELLIOTT, C.J.: What other techniques have you tried for the inactivation of these "command" neurons? (e.g. photoinactivation, injection of protease).

SYED, N.I.: What criterion have you used to justify your "command neurons"?

BALABAN, P.M.: For investigation of command function of a certain neuron we have used intracellular activation, reversible ablation by means of hyperpolarization, and recorded their activity during adequately evoked behaviour. It was found that

these cells respond to the criteria suggested by I. Kupfermann and K. Weiss.

KEMENES, G.: 1. In the behavioural experiments you paired food stimulus with electric shock. In the semi-intact preparations, you used quinine as aversive stimulus. Did you try to use electric shock on semi-intact preparations the same way you did in intact animals?

2. In my opinion (supported by our experiments on Helix and Lymnaea) 5,6-DHT (and possibly 5,7-DHT, too) has an effect on the feeding behaviour itself. Does this not make the explanation of the effect of these drugs on learning more complicated?

BALABAN, P.M.: 1. Yes. We have used in semi-intact preparations electric shock as well as quinine and 5-HT.

2. We have noted the effect of 5,7-DHT treatment only on appetitive phase of feeding behaviour. Consummatory phase (triggered by dopamine) remained intact.

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NEURONAL MECHANISMS OF LEARNING IN APLYSIA: MORE THAN JUST SENSORY-MOTOR SYNAPSES ARE INVOLVED

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"What goes on in my brain when I learn something?" and "How do I remember things?" are two questions commonly asked by the person sitting next to me on the airplane when they find out that I'm a neuroscientist. People are very interested in how the brain works; they want to know how we know. My seatmate knows that the human brain is far too complex a structure in which to ask these questions so they assume, rightly, that the work must be done on less complicated organisms. What most of them do not realize is that invertebrate model systems are providing some of the best information as to what the neuronal mechanisms of learning and memory are. Many invertebrates are capable of associative learning and this literature has been extensively reviewed (Mpitsos and Lukowiak, 1985; Byrne, 1987).

Of all these model systems, one of the most detailed hypotheses for the cellular and molecular basis of learning has emerged from the studies on Aplysia californica. By far the best studied behavior in this model system has been the gill withdrawal reflex (GWR). It needs to be stated at the outset that the GWR is not simply a graded contraction dependent upon the stimulus strength as stated by Kandel (1976) but rather is a heterogeneous collection of at least 4 action patterns which persist even after the removal of the parieto-visceral ganglion (PVG) or abdominal ganglion - that part of the CNS which innervates the mantle organs of the animal (Leonard et al, 1987). The contribution that each of the four action patterns makes may change

during the course of learning and this will certainly have to be studied in the future.

The GWR evoked by tactile stimulation of the siphon or gill undergoes two types of non-associative learning - habituation and sensitization (Pinsker et al, 1970; Pinsker et al, 1973). Accompanying these behavioral changes are changes in the evoked synaptic input to the central gill motor neurons which correlate well with the observed behavioral changes. Whether these observed cellular correlates are both necessary and sufficient for the observed behavioral changes, are still a matter of some controversy, since the contribution of the peripheral nervous system (PNS) between the siphon and the gill was ignored in those studies. The PNS and CNS interact and form an integrated system in the mediation of adaptive gill reflex behaviors (Lukowiak and Peretz, 1977). Changes which occur within the PNS itself, also play a major role in the mediation of these non-associative learning phenomena.

Changes in synaptic efficacy at the sensory-motor neuron synapse may underlie some of the behavioral changes (Kandel and Schwartz, 1982). The cellular correlate of habituation is low frequency monosynaptic depression which may be due to a progressive inactivation of a Ca^{++} current and depletion of neurotransmitter substance (Gingrich and Byrne, 1985, 1987). Sensitization has been correlated with an increased Ca^{++} influx, due to a decrease in a specific K^{+} current (Ks) and to a mobilization of transmitter stores (Gingrich and Byrne, 1985, 1987). Some of the changes described above may involve increasing levels of cyclic AMP and altered protein synthesis (Montarolo et al, 1986). It must be remembered that many of these studies are correlational; whether these changes are both necessary and sufficient for the behavioral changes still remain to be considered.

The GWR can be classically conditioned both in the intact animal (Carew et al, 1983) and in <u>in vitro</u> preparations (Lukowiak and Sahley, 1981; Lukowiak, 1986; Colebrook and Lukowiak, 1987). As well simulation of the classical conditioning paradigm in isolated ganglia preparations produces neural correlates of the associative learning phenomena (Hawkins et al, 1983; Walters and Byrne, 1983). This neural correlate, activity dependent neuromodulation (Walters and Byrne, 1983) explains

quite well the temporal specificity important for classical conditioning.

Thus it would appear that the neuronal mechanisms of classical conditioning of the GWR in Aplysia have been worked out. However, there is a problem; only correlational studies have been performed. It has not been demonstrated that these changes actually occur as the behavior changes or that they are necessary and sufficient for the associative learning.

Lukowiak (1986) and Colebrook and Lukowiak (1987) using two different in vitro preparations found that although changes did occur at the sensory-motor neuron synapse as described, these changes were not necessary or sufficient to account for the observed behavioral changes. Thus the hypothesis was put forward which states that classical conditioning training leads to changes in the activity of CNS control neurons (see Lukowiak and Peretz, 1980) which regulate the ability of gill motor neurons to elicit a gill withdrawal response. If the above hypothesis is correct, then the ability of a gill motor neuron to elicit a gill withdrawal response, should be altered following classical conditioning. Thus, if the same number of APs are evoked in the gill motor neuron before and after learning, the elicited gill withdrawal response should be larger following classical conditioning training. This hypothesis was tested in two preparations. It was found that classical conditioning training affected the ability of the gill motor neuron to elicit a gill withdrawal response and thus support for this hypothesis was obtained.

METHODS

Aplysia californica obtained from SeaLife Supply (Sand City, California) weighing between 100-300 gms were used in these studies. They were maintained in 1200 litre aquaria (artificial sea water, ASW; instant ocean) maintained at 15-17^{o}C, pH 7.9. Animals were fed dry red seaweed once a week. Food satiated Aplysia, which show suppressed gill behavior (Lukowiak, 1980), were not used in these studies. Animals were anesthetized, isotonic (0.33 M) $MgCl_2$ prior to dissection.

One of the semi-intact preparations consisted of a siphon, mantle gill, PVG, circumesophageal or head ganglia (cerebral, pedals and

pleurals) and the tail with its innervation intact. The siphon, branchial and ctenidial nerves were left intact, as were the pleural-abdominal connectives and the two posterior pedal nerves. All of the other nerves and connectives were severed. The preparation was pinned, dorsal side down to a Sylgard (Dow Corning) base. The PVG and head ganglia were further pinned out on clear Sylgard platforms. This preparation will be referred to as the "Colebrook" preparation. The other preparation was similar to the first except that only the PVG's innervation of the siphon, mantle and gill was left intact. All other nerves and connectives were severed. This preparation will be referred to as the "Lukowiak" preparation. Gill tension was measured with a force transducer.

Central gill motor neurons, L7, LDG_1 and LDG_2, were identified according to the type of gill movement they elicited when depolarized (Leonard et al, 1987).

In the "Colebrook" preparation, the conditional stimulus (CS) consisted of a weak (600 mg) tactile stimulus delivered to the siphon by a "tapper". This stimulus normally evoked an EPSP in the gill motor neuron. This CS is similar to that used in the intact animal studies (Carew et al, 1983). The unconditional stimulus (UCS) was a train of shocks to the pedal nerve (similar to that used in the Hawkins et al, 1983 study) delivered via bipolar silver hook electrodes. In the preparations classically conditioned, the CS preceded the UCS by 500 msec and the intertrial interval was 5 minutes. In the control group, the CS and the UCS were specifically unpaired; the UCS presented 2.5 min after the CS. Ten training trials were given in each condition.

In the "Lukowiak" preparation, the CS was similar to that of the "Colebrook" preparation, while the UCS was a train of tactile stimuli (1.5 g, 8 per sec for 1 sec) applied to the gill. In the classical conditioning group, the CS preceded the UCS by 500 msec and the intertrial interval was 2 min. Control group preparations were also specifically unpaired, the UCS presented 1 min after the CS.

The ability of the gill motor neuron to elicit a gill withdrawal response was monitored before and after training. The motor neuron was depolarized to produce a given number of APs for 2 sec; 20 minutes later, the motor neuron was again depolarized to produce the same number

of APs. Experiments only proceeded if the amplitude of the elicited gill responses were similar. In the "Colebrook" preparation the ability of the gill motor neuron to elicit a withdrawal response was tested 5 min after the CS only was presented (30 min after the 10th CS-UCS trial course) while in "Lukowiak" preparation, the motor neuron was tested 30 sec after the last CS-UCS trial.

Significance was determined by a t-test.

RESULTS

The "Colebrook" preparation can be classically conditioned (Colebrook, 1987; Colebrook and Lukowiak, 1987). The ability of a gill motor neuron to elicit a gill withdrawal response 35 min after the last conditioning trial was determined, 3 different outcomes were observed. In the control group (i.e. specifically unpaired CS-UCS), 86% showed a decrease in the amplitude of their elicited withdrawal response. Only one preparation elicited a larger response. In the classical conditioning training group, on the other hand, 54% of the preparations showed either no change or an increased withdrawal response.

When the data were combined together, the unpaired (control) group showed a mean decrease in the response amplitude to motor neuron depolarization of 50%. The paired group demonstrated a slight mean decrease of only 7% (Figure 1). Unpaired training resulted in significant decrement of elicited gill withdrawal response ($p<0.05$); while in the paired group there was no significant change observed ($p>0.05$). Unpaired training, therefore, causes a decrement in the ability of the motor neuron to elicit a gill withdrawal. Such a decrement is not evident after the classical conditioning training period.

A typical example of the data obtained from the "Lukowiak" preparation are shown in Figure 2.

The effect of the presentation of both paired and unpaired stimuli can be seen on the motor neuron's ability to elicit a gill response. Following the two control depolarizations of LDG_1 (T_1 and T_2), 10 specifically unpaired stimuli were presented. When LDG_1 was again depolarized (T_3), it produced a smaller gill withdrawal response. The

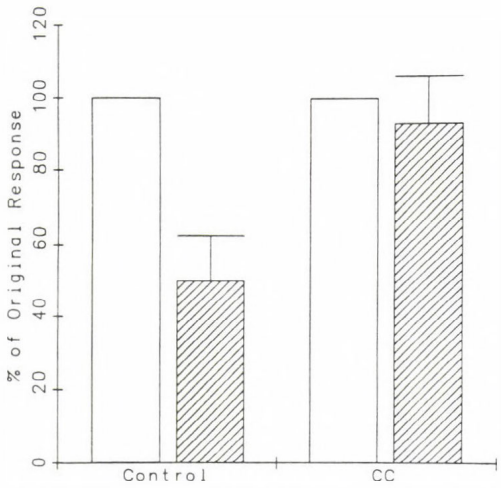

Figure 1. Mean amplitudes of the gill withdrawal responses elicited by depolarization of the gill motor neuron in the "Colebrook" preparation. The response elicited after 10 trials (post) was calculated as a % of that elicited before training (pre). Error bars are the S.E.M.

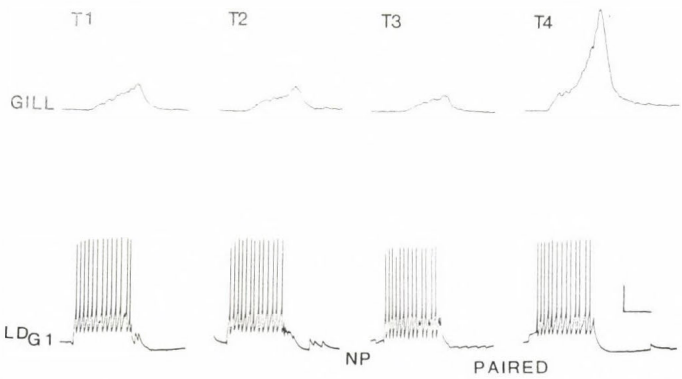

Figure 2. LDG_1 was depolarized at an ISI of 20 min to obtain the control response amplitude (T_1, T_2). Following T_2, the preparation received 10 specifically unpaired stimuli (NP) and 30 sec after the last trial (T_3), LDG_1 was again depolarized. This resulted in a smaller response. The preparation was rested for 1 h and then it received 10 paired stimuli (paired). When LDG_1 was now depolarized (T_4) it produced a much longer response. Scale: 1 sec; 20 mv.

preparation was then rested 1 hour before receiving paired stimuli. When (T_4) LDG_1 was again depolarized, a much larger gill response was observed. Data from a second preparation show the effect of both increased training and the decay of the facilitation with rest (Figure 3). Data similar to these were obtained with LDG_2 and L_7 (not shown). When all the data were combined, it was found that on average (n=7) following the specifically unpaired stimuli presentations there was a 41.6% (± 12.2) decrease in the gill response produced by motor neuron depolarization. However, following paired presentation of the stimuli (n=8) there was a 342% (± 88) increase in the gill response produced by motor neuron depolarization. Both changes were significant at the $p<0.05$ level.

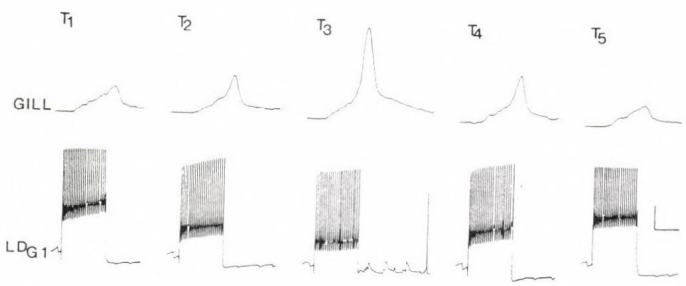

Figure 3. T_1 is the second control depolarization. Following 10 paired stimuli, depolarization of the LDG_1 (T_2) leads to a larger gill response. Following a further 10 paired stimuli the response (T_3) was even larger. When tested 30 min later (T_4), the withdrawal response was smaller. Following another 30 min rest (T_5), the response was similar to control. Scale: 1 sec; 20 mv.

DISCUSSION

Classical conditioning of the gill withdrawal reflex has been shown to occur in both intact Aplysia (Carew et al, 1983) and in three different in vitro preparations (Lukowiak and Sahley, 1981; Lukowiak, 1986; Colebrook and Lukowiak, 1987). In addition, in the isolated ganglia preparation of Aplysia, simulations of classical conditioning training result in changes in synaptic efficacy at the central sensory-

gill motor neuron synapses (Hawkins et al, 1983). Based on correlational data between the intact Aplysia studies and the isolated ganglia studies, it has become accepted (Byrne, 1987) that the changes in synaptic efficacy which occur at the sensory gill motor neuron synapse constitute a mechanism of associative learning. However, the in vitro preparation studies where behavioral and cellular changes can be monitored simultaneously, show that while changes in synaptic efficacy do occur, as earlier described, they are neither necessary nor sufficient for associative learning. Thus, the hypothesis was proposed that classical conditioning training leads to changes in the activity of central control neurons which regulate the ability of gill motor neurons to elicit a gill withdrawal response.

Two different in vitro preparations were used to test this hypothesis, the "Colebrook" preparation and the "Lukowiak" preparation. The results obtained from both preparations tend to support the proposed hypothesis. However, there were some differences in the results obtained from the two preparations but these differences may be due primarily to the experimental design used in the "Colebrook" preparation. In those experiments, the efficacy of the motor neuron was tested 35 minutes after the last presentation of the paired stimuli. This was necessitated by the fact that learning was assessed by determining how the preparation responded to the CS presented 30 minutes after the last CS-UCS stimuli (Colebrook, 1987; Colebrook and Lukowiak, 1987). Thus the ability of the gill motor neuron to elicit a gill movement could only be assessed 5 min after the presentation of the CS. Also, only 10 paired trials were given. As shown in Figure 3, using the "Lukowiak" preparation, the heightened motor neuron efficiency decreased within 30 minutes. Thus, it is not too surprising that the data from the "Colebrook" preparation show little or no change in the motor neuron efficacy to elicit a gill movement following classical conditioning training. However, when these results are compared to those obtained with a specifically unpaired paradigm, there are clear and significant differences. The efficacy of the motor neuron to elicit a gill withdrawal response is significantly depressed when the stimuli are not paired. Thus, classical conditioning training affects in a facilitory manner, the motor neuron's efficacy to cause a gill withdrawal response.

The data obtained with the "Lukowiak" preparation were much more clear cut. There it was shown that the ability of a motor neuron to elicit a gill withdrawal response was facilitated significantly following classical conditioning training. Three different motor neurons were tested and all gave similar results. On the other hand, in control preparations, the ability of the motor neuron to elicit a gill response was significantly depressed. This could even be seen in the same preparation (Figure 2) where the efficacy of the motor neuron was decreased following unpaired presentation of the stimuli but became facilitated when the stimuli were paired.

It is hypothesized that the pairing of the CS-UCS leads to an increase in activity of CNS control neurons (Lukowiak and Peretz, 1980) which mediate facilitation. While with the unpaired stimuli, the neurons which mediate suppression increase their activity. That these as yet, unidentified control neurons can be differentially activated has recently been documented (Lukowiak and Colmers, 1987). In that study, the ability of a motor neuron to elicit a gill withdrawal response was affected by the superfusion of endogenous peptides over the PVG. For instance, SCP_B superfusion, facilitated the ability of the gill motor neuron to elicit a gill withdrawal response, while AVT suppressed the response. Since the peptides were only superfused over the PVG and had no effect on the passive membrane properties of the motor neurons, they could only have worked via the central control neurons.

In addition to those results, peptide perfusion of the gill of Aplysia has also been shown to significantly affect the GWR evoked by gill or siphon stimulation. This demonstrates that activation of neurons in the periphery can affect the behavior of the gill. Cawthorpe and Lukowiak (1987) have demonstrated that FMRFamide perfusion of the gill significantly facilitates the GWR and prevents its habituation while SCP_B perfusion has the opposite effect (Cawthorpe and Lukowiak, 1986). Activation of CNS control neurons by different stimuli, such as classical conditioning, could lead to the release of neuroactive agents in the PNS and thus affect how the gill will respond to the various stimuli.

Obviously, many more experiments will need to be performed before it can be certain that associative learning involves activation of

central control neurons which facilitate the ability of the gill motor neurons to elicit a response. However, the data so far obtained support this notion and are consistent with the data obtained earlier from the in vitro experiments which demonstrate that changes in synaptic efficacy at the sensory motor neuron synapse are not themselves sufficient and necessary for learning to occur.

The neuronal mechanisms which underlie associative learning in the Aplysia preparation involve changes in activity at a number of different loci, both within the PVG and the PNS and any analysis of the neuronal mechanisms which underlie learning, all the loci must be taken into account.

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DISCUSSION

BALABAN, P.: In our experiments excitability changes occurred in premotor neurons during elaboration of plastic changes. We have some evidence that these changes of excitability are elicited by peptides. Have you checked excitability in motoneuron LDG-1?

LUKOWIAK, K.: I have not tested this yet in a systematic manner but the preliminary results show that it takes less current to evoke the same number of APs following conditioning training.

SAKHAROV, D.A.: Could you tell us something on the chemical nature of neuromuscular transmission in the gill? The transmission was claimed dopaminergic in the literature. We could not, however, reveal dopamine in cell bodies of gill neurons in the abdominal ganglion.

LUKOWIAK, K.: The neurotransmitter of the sensory neurons in the PVG is not yet known. In the gill, dopamine causes a contraction and so does ACh and FMRFamide. The neurotransmitter used by L7 is not known. Cell L9 which is itself probably not dopaminergic does appear to cause the liberation of DA in the gill. We are not certain how this happens. The PNS in the gill is quite complex and does play a major role in the mediation of adaptive gill behaviours. The CNS and PNS interact. The changes which occur in the PNS during learning have not yet been examined.

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DEMONSTRATION OF THE ADULT-SPECIFIC FLIGHT MOTOR PATTERN IN ISOLATED GANGLIA AND ALL INSTARS OF LOCUSTA, BY USE OF OCTOPAMINE

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INTRODUCTION

The motor pattern underlying the locust flight performance is a well studied system. In his now classical paper, Wilson (1961) presented the first evidence for an innate central pattern for the production of flight movements in the locust. At this time peripheral feedback from the moving wings was thought to control only the average wing-beat frequency (Waldron 1967a; Kutsch 1974). Later, however, it was shown that peripheral sense organs can exert a phasic cycle for cycle influence on the flight pattern (Wendler 1972), and claimed that such loops should be considered as integral parts of the oscillating network (Altman 1983; Wendler 1983; Pearson et al. 1983). These studies, together with recent investigations which indicated that the rhythmic activity generated by functionally deafferentated locusts (Robertson & Pearson 1982) is not simply a slowed down version of the normal flight pattern (Hedwig & Pearson 1984; Pearson & Wolf 1987), finally led some authors to question the validity of the central pattern generator (CPG) concept for locust flight (Pearson 1985; Wolf & Pearson 1987a).

The strongest evidence for central control of motor patterns comes from studies in which all or part of the CNS are isolated from the rest of the animal (Delcomyn 1980). This has never been satisfactorily shown for locust flight since wind stimulation of head receptors is usually required to initiate a flight motor response. However, this problem can be circumvened by exploiting a recent technique involving the neuromodulator octopamine: which has been shown to release long bouts of flight activity when ionophoretically injected into discrete regions of the metathoracic ganglion

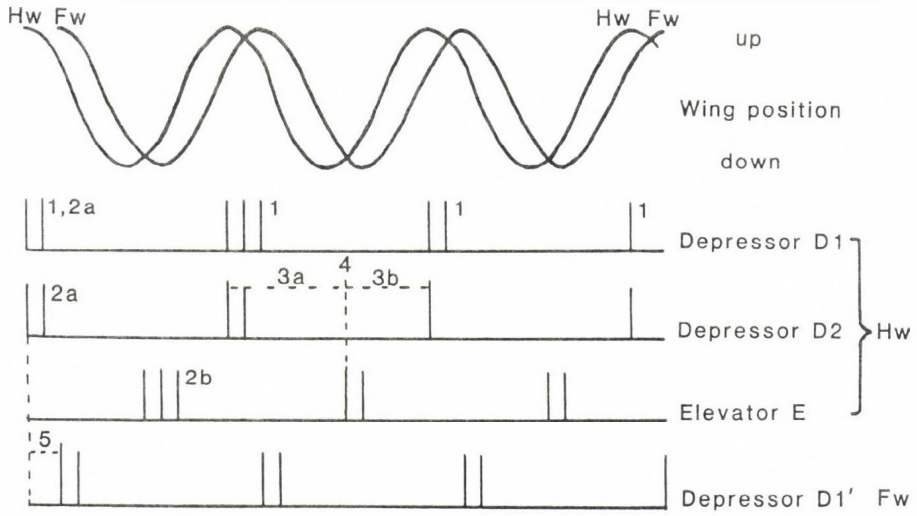

Fig. 1: Correlation of wing position (Hw - hindwing, Fw - forewing) and motoneuronal activity during locust flight. The graph illustrates the different "Features of the normal flight motor pattern" as presented in the text

(Sombati & Hoyle 1984b) or simply applied to the nerve cord (Stevenson & Kutsch 1987a). The present investigation will demonstrate the capacity of isolated ganglia to generate a motor output which has all major features in common with the normal flight motor pattern. We also used this biogenic amine to demonstrate the basic flight circuitry in all larval stages.

FEATURES OF THE NORMAL FLIGHT MOTOR PATTERN

There are several features describing the flight motor pattern in intact tethered locusts (Wilson & Weis-Fogh 1962; Waldron 1967b; Kutsch & Stevenson 1984; Stevenson & Kutsch 1987a). These might be used as reference for any released motor output (s.Fig.1).

1) The motor units are recruited at the same overall frequency (for Locusta ca. 20 - 25 Hz), usually once or a few times per wing-beat cycle (1,D1).

2) The different motor units of a segment split up into two functional alternating groups → depressors (2a,D1-D2) and elevators (2b,E); contralateral homologous muscles are activated in near synchrony (2a,D1-D2).

3) The latency D-E (3a) is longer than the reverse interval E-D (3b).

4) Both, E-D- and D-E-latencies increase proportionally with increasing cycle length. As a result the phase of E-motor units within the D-activity cycle ($4 \rightarrow \phi = 3a / 3a + 3b$) remains relatively constant. For Locusta a value of about 0.55 to 0.6 is found normally, indicating that the period of the downstroke is slightly longer than the upstroke.

5) Motor units of the hindwings are activated several ms in advance of their mesothoracic serial homologues.

RELEASE OF FLIGHT MOTOR ACTIVITY IN ADULTS WITH OCTOPAMINE

In search for the minimal circuitry which is able to generate a specific motor pattern the system has to be reduced as far as possible. Such studies require a technique to initiate a motor response independent of the normal peripheral stimuli. For flight usually wind stimulation of head receptors is required. Previously, electrical shocks to the nerve cord have been applied (Wilson 1961; Wilson & Wyman 1965). However, these studies never reached the level of single ganglia. We, therefore, turned our attention to a recent technique by applying octopamine to the CNS. Sombati & Hoyle (1984b) reported that flight similar motor activities can be evoked by ionophoretically releasing this neuromodulator into discrete regions of the metathoracic ganglion in Schistocerca americana. However, two problems remained: 1) Since details of the pattern were not reported it remained uncertain as to what extent the octopamine released activity corresponded to the basic motor pattern. 2) Since these previous experiments were carried out on minimally dissected locusts, the possibility remained that peripheral feedback loops are required to facilitate the response.

Flight motor activity in functionally deafferentated locusts

Initially, we injected octopamine into the thoracic ganglion of the fully deafferentated locust (Stevenson & Kutsch 1987a). As described by Sombati & Hoyle (1984b), the position of the micropipette within the ganglion was a crucial factor. Flight motor activity was only released when a region was impaled approximately corresponding to the position "2" described by Som-

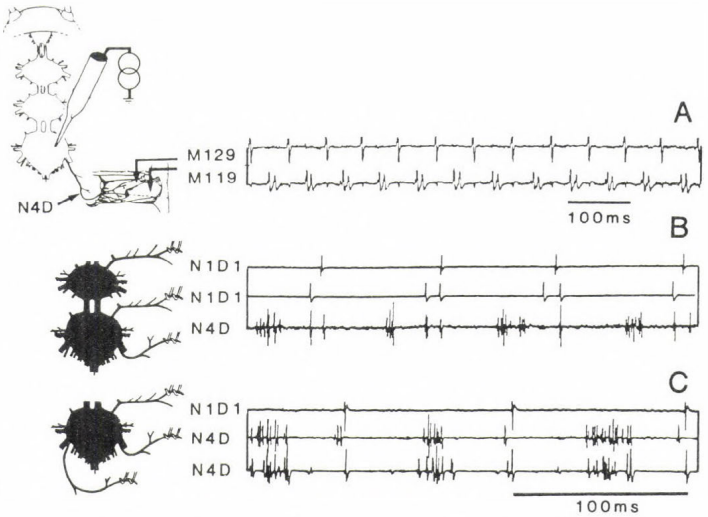

Fig. 2: Octopamine released flight motor pattern for different levels of CNS isolation. **A** Ionophoretical application to the metathoracic ganglion of a deafferentated preparation. Electromyographic recording of a depressor (M 129) and elevator (M 119) activity in the metathorax. **B** Topical application to an isolated meso-metathoracic ganglia complex. Released activity has been documented by recordings from stumps of severed motor nerves containing the axons of either depressor (N1D1) or both depressor and elevator motoneurones (N4D). Note the typical time shift between homologous motor units of the fore- and hindwings. **C** Topical application to an isolated metathoracic ganglion. Recording procedure similar to **B** (modified from Stevenson & Kutsch 1987a)

bati & Hoyle (1984b). Often alternating rhythmic activities of longer duration could be released (Fig. 2A) which continued even with the current to the pipette turned off.

As a control experiment we employed a double-barrelled micropipette (Stevenson & Kutsch 1987b). Hereby, the effects of both current and octopamine injection could be compared independently and it could be tested whether the response was caused simply by electrical stimulation in this specific region. For current injection alone no response resulted. However, octopamine at the same site repeatedly evoked a flight motor sequence implicating this substance as the effective agent. The amount of octopamine (due to longer current injection) and the duration of the flight performance are related to each other.

Essentially the same result could be achieved by applying octopamine topically to the thoracic ganglia (Stevenson & Kutsch 1986,1987a). Apparently the ganglionic sheath is a formidable barrier to this substance since the effects were only seen with rather high concentrations (0.1 M). Even though for many aspects, especially those devoted to studies of isolated ganglia or the development of the motor pattern (s. later), this method of topical application rendered suitable results. Recently octopamine was also applied topically to the ganglia of near intact mature animals. This apparently resulted in a normal flight motor output which was not drastically changed when combined with a wind stimulus (unpublished observations).

Analyses of these responses demonstrated major features of the basic flight motor pattern. The overall output frequency was in the range of that expected by deafferentated preparations (10 - 12 Hz) but could also reach higher values (up to 17 Hz). The frequency remained rather steady throughout any one sequence. With a few exceptions the interval D-E was longer than the reverse interval and the phase of E within the D-cycle remained relatively constant independent of the cycle length; this phase constancy is a typical feature of the normal flight (Waldron 1967b; Kutsch & Stevenson 1984).

Flight motor activity released from isolated ganglion preparations

The next step in search for the minimal circuitry generating the basic flight motor pattern was to isolate the meso- and metathoracic ganglion complex. For this we dissected this complex out of the animal and transferred it to a bath to which octopamine was topically applied (Stevenson & Kutsch 1987a).

By recording from specific nerve stumps a motor output was detected which had all major features in common to the normal flight pattern (Fig. 2B). Even the characteristic meta-mesothoracic time lag for serial homologous units (which is the basis of the normal leading of the hindwings vs. the forewings) is seen in such a preparation; this argues that this time lag is rather more dependent on central interactions than on peripheral control.

Previous investigations had suggested that the flight pattern generator is a single entity distributed among several ganglia and operating as a unit (Robertson & Pearson 1984; Wolf & Pearson 1987b). This assumption was tested by applying octopamine to the isolated ganglion. At least, for the metathoracic ganglion a pattern could be evoked (Fig. 2C) which in all major aspects corresponded to the basic flight motor pattern (Stevenson & Kutsch 1987a). To date we have not yet been able to release a similar pattern from the fully isolated mesothoracic ganglion. This might indicate that either there is no equivalent set of neurones to the metathorax or these neurones need a specific input from the metathoracic ganglion. However, after cutting the meso-metathoracic connectives in an otherwise intact animal a typical antagonistic activation of the forewing muscles can still be evoked by wind blown on the head (Stevenson & Kutsch 1987a). We propose therefore, that each pterothoracic ganglion has a full complement of neurones generating the basic flight motor pattern. The difficulty in releasing a flight pattern in the isolated mesothoracic ganglion may indicate that inputs from the metathoracic ganglion normally are involved in the processes for pattern release.

RELEASE OF THE FLIGHT MOTOR PATTERN IN ALL LARVAL STAGES

This biogenic amine also enabled us to study the development of a specific behavioural pattern. It should be noted that we here deal with a motor pattern the execution of which is bound to the adult stage, i.e. to the unfolding of the freely moving wings. Is this of any consequence for the formation of the relevant neuronal circuitry such that larval-adequate motor patterns must be completed with embryogenesis while adult-specific ones will be constructed only later?

Previously it had been claimed that with the imaginal ecdysis there is a dramatic switch in the motor output when tethered in the wind stream (Kutsch 1971; Altman 1975): while adults of any age immediately generate the typical rhythmic flight motor pattern (whereby the wing-beat frequency increases with maturation) there is only an irregular tonic pattern in larval stages. Incidentally, these larval stages adopt the characteristic "flight position" (it has been speculated that this posture actually repre-

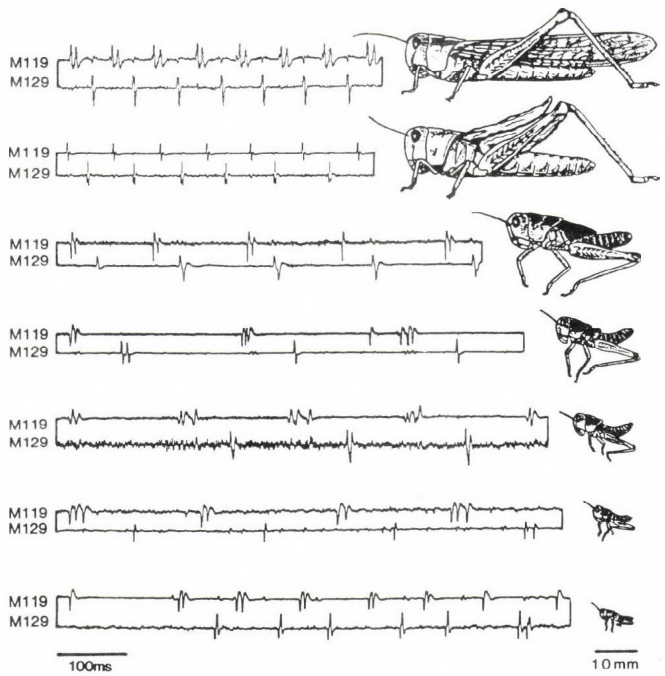

Fig. 3: Flight motor activity released by octopamine in all stages of Locusta. Electromyographic recording of a metathoracic pair: elevator (M 119) and depressor (M 129). Top to bottom: mature adult, newly moulted adult, 5th to 1st larval instar. For the preparational state of the different stages and application procedure, see Stevenson & Kutsch (1987a, Fig. 7)

sents the general "jumping position" which becomes incorporated into the flight performance after imaginal ecdysis ; Kutsch 1983). The major developmental event for this switch appeared to be the imaginal ecdysis. If due to hormonal manipulations the imaginal moult was induced to occur at an earlier stage than the resultant precocious adult responded to wind by generating the rhythmic flight pattern (Kutsch & Stevenson 1984). It has been assumed that within a few days around last ecdysis some specific neuronal reconstructions including possible ingrowth of peripheral axons occur which result in the remarkable change of motor output. This idea, however, was questioned by later experiments which showed that last larval instars can occasionally be induced to generate flight similar rhythmic activity (Kutsch 1985), after prolonged stimulation of the larvae with wind. Since it took such a long time to start rhythmic activity it was excluded that normal neuronal interactions were responsible but rather that hormonal factors may be involved in flight initiation and maintenance. As a possible candidate octopamine was favoured which is becoming recognized as a neuro-

modulator in insects (David & Coulon 1985). The concentration of this substance within the brain increases in parallel to aging of the last larval Locusta (Fuzeau-Braesch et al. 1979). Similarly, a change in the sensitivity of adenylate cyclase to octopamine with the imaginal ecdysis was reported (Hiripi & S.-Rózsa 1984). Furthermore, it has been demonstrated that octopamine concentration in the haemolymph will increase due to unspecific stimulation ("handling" or "stress"; Bailey et al. 1984; Davenport & Evans 1984). A continuous air flow over the animal's head and body could be equivalent to a "stress" situation and in combination with a raising of the octopamine level a greater sensitivity of older larval stages to flight inducing stimuli might result. From this it might be inferred that decisive processes in the formation of the flight motor circuitry are not bound to the time of imaginal ecdysis. The normally observed unpatterned activity of the larval instars could also indicate the inability to respond to the wind with a proper activation of the flight motor centre. The neuronal network for the flight motor pattern might have been formed much earlier.

To test this hypothesis we applied octopamine to different larval stages (Stevenson & Kutsch 1987b). While a preparation of a 5th (= last) larval stage will respond with a typical tonic (= larval) pattern when exposed to a wind stream it might show a clear flight motor pattern (Fig. 3) when octopamine is applied either ionophoretically or topically. As is

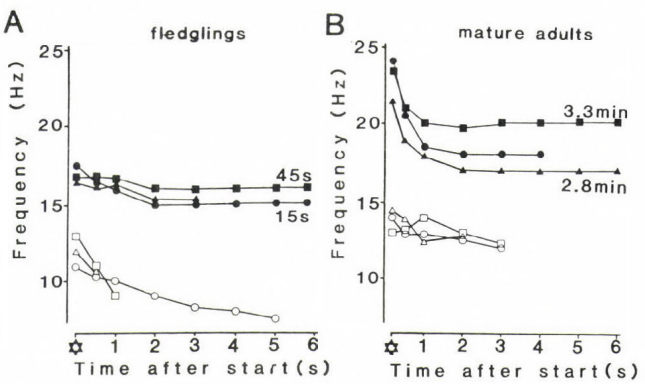

Fig. 4: Enhanced response of deafferentated adult locusts to wind stimulation following octopamine ionophoresis. Sequential plots of the frequency of wind released flight motor sequences from start onwards (* instantaneous frequency of first depressor cycle) for 3 fledglings **A** and 3 mature adults **B**. Open symbols: before, filled symbols: after octopamine ionophoresis (modified from Stevenson & Kutsch 1987b)

characteristic of the normal flight pattern metathoracic depressor motor units were found to be activated several ms in advance of their mesothoracic homologues.

All the earlier larval stages were similarly tested. Although it became progressively more difficult, we finally succeeded to release the flight motor pattern in all instars. The frequency ranged from 5 to 12 Hz and even the first larval stage could exhibit a rhythmic output similar to that recorded for adult fledglings.

MODULATION OF MOTOR ACTIVITY BY OCTOPAMINE

Due to results with Manduca sexta, Kinnamon et al. (1984) suggested that octopamine acts centrally by altering the level of excitation or effectiveness of synaptic transmission. Sombati & Hoyle (1984a) had demonstrated a central enhancement of a reflex activity in locusts. In search for possible central processes affected by octopamine we tested the interaction of this biogenic amine with a flight performance released by natural stimulation (wind to the head). In the deafferentated preparations wind would release only a weak flight performance. However, when octopamine was applied additionally a flight motor output resulted which was superior to any other reported activity of such a preparation. Occasionally, frequencies of 20 to 24 Hz were observed which remained stable for more than 3 minutes; such performances approached those of intact locusts (Fig. 4B).

With fledglings analogous experiments were carried out (Stevenson & Kutsch 1987b). Octopamine not only released long motor sequences which exhibited typical features of the flight motor pattern (Fig. 3), but also an interaction of both, wind stimulus and octopamine was seen. With a combination of both stimuli a flight response resulted which was comparable to the performance of intact fledglings in the wind tunnel (Fig. 4A). Thus, under these specific circumstances the difference in the motor output frequency between the young and mature adults remained such as is typical for normal tethered flight (i.e. the wing-beat frequency increases with maturation; Kutsch 1971,1973).

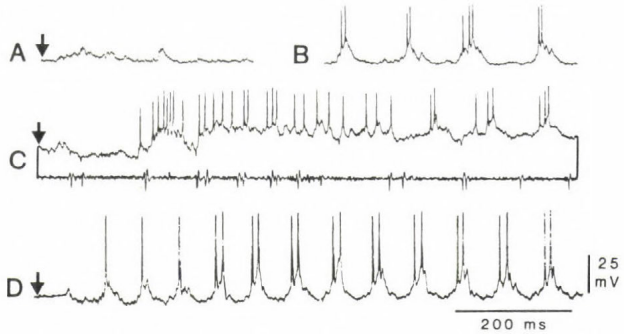

Fig. 5: Intracellular recording from a depressor motoneurone of a deafferentated 5th larval instar illustrating the enhanced response to wind stimulation (arrow) following octopamine treatment. **A** response to wind before octopamine ionophoresis. **B** Octopamine released flight motor sequence. **C** Enhanced response to wind following octopamine ionophoresis; for reference an extracellular recording from an elevator muscle is given in the lower trace, illustrating a brief sequence of neuronal alternation. **D** Recording of wind released activity of the same motoneurone from a deafferentated mature adult locust (from Stevenson & Kutsch 1987b)

An interaction between octopamine and central activity was also demonstrated for a last larval instar. Wind alone could release only a short irregular activation of a flight motoneurone. Application of octopamine, however, would alter the wind response towards a flight motor output (Fig. 5).

CONCLUSIONS

It is clear now that isolated thoracic nerve cord preparations can generate a motor activity which seems to represent the basic flight motor pattern; i.e. a motor pattern incorporating all major aspects of a normal tethered flight performance (s. Fig. 1 and corresponding text), but at a reduced output frequency. This motor programme can be generated by the fully isolated metathoracic ganglion. There is some evidence that the mesothoracic ganglion may have a similar property. Furthermore, it is concluded that major steps in the development of the flight motor circuitry are completed by the end of embryogenesis. Post-embryonic changes neither centrally nor peripherally seem to be of great importance for the ontogeny of the locust flight motor programme itself. However, at imaginal ecdysis there might happen some developmental changes in the wind sensory system and/or changes in the level of neuromodulators (such as octopamine) which might be involved in the activation of the flight motor programme.

Further studies have to reveal the importance of octopamine or an equivalent neuromodulator in pattern generation. Where is it produced? Where is its releasing site? How does it interact with the flight motor circuitry? What is the actual role of the octopamine containing DUM cells (Sombati & Hoyle 1984b)? Why does the locust develop a complete circuitry such early that can be functional only with last ecdysis, i.e. with the unfolding of the freely moving wings? Though the relevant flight muscles exist from late embryogenesis onwards this specific pattern does not appear to serve any other behavioural action. Is there an active inhibition of the programme as proposed for crickets by Bentley & Hoy (1970), or is its activation mainly dependent on the concentration of a specific neuromodulator which level can be regulated during development?

ACKNOWLEDGEMENT

The original research on the ontogeny of the locust flight was continuously generously supported by the Deutsche Forschungsgemeinschaft. The octopamine studies were made possible especially by the DFG-grant Ku 240/13 2,3.

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DISCUSSION

HONEGGER, H.-W.: If I remember right Sombati and Hoyle injected octopamine into the metathoracic ganglion and initiated walking. When you expose the whole ganglion in a high concentration of octopamine a lot of circuits (walking, flying, etc.) would be switched on. Don't you observe more rhythmic motor outputs than just flying in your preparation?

KUTSCH, W.: The usual released pattern is the flight motor pattern when octopamine is applied topically. This observation lets us speculate that for the release of this motor pattern the lowest threshold has to be passed. This might have to be in correlation to the normal escape response. For the adult locust it is the safest to take off and fly away with any immense stimulation.

MOFFETT, S.: Am I correct in reading your slide that the concentration of octopamine that you apply is 0.1 M?

KUTSCH, W.: Yes. Apparently the ganglionic sheath is a formidable barrier for this substance, which will decrease the central octopamine concentration considerably.

PENZLIN, H.: Octopamine is known as an excitatory and as an inhibitory transmitter as well in insects. What is in your opinion the real function of octopamine? Does octopamine activate the motor system you spoke about or is it a disinhibition?

KUTSCH, W.: It is too early to speculate about a specific central action of octopamine in our system. We hope that further studies will reveal the receptor site for this substance and then we might be able to understand the interaction of the neurons involved.

SAKAI, M.: How long does the octopamine effect last after iontophoretic application?

KUTSCH, W.: There is a correlation between the duration of the octopamine pulse and the flight performance. Occasionally such a response could last for minutes.

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MODULATION OF PRIMARY SENSORY NEURONS AND ITS RELEVANCE TO BEHAVIOUR IN THE POND SNAIL LYMNAEA STAGNALIS

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Many sensory organs receive efferent innervation. In vertebrates this holds especially for the retina and the vestibular apparatus. The complexity of these sensory organs makes it often difficult to study the function and mechanisms of efferent innervation. In *Lymnaea* and other gastropods the statocysts are relatively simple structures, they contain about 13 sensory cells (Geuze, 1968; McKee and Wiederhold, 1974; Wolff, 1975; Janse, 1983). Moreover, in a number of gastropods efferent innervation of the statocysts has been reported(Wolff, 1970; 1975; Alkon et al., 1978). This makes these animals suitable objects to study the role of efferent innervation in sensory processing and behaviour. For most of these animals, however, there are no indications about a possible function of efferent innervation in behaviour. *Lymnaea*, however, seems of special interest because changes in gravity orientation can be induced under particular conditions. Behavioural experiments have shown that gravity orientation in *Lymnaea* is important for respiration and is influenced by the respiratory condition of the animal. In O_2 - rich conditions (after breathing) the animal is positively geotactic and moves downwards. In O_2 - poor conditions the animal displays negative geotaxis and moves to the water surface to breath air (Janse, 1981; 1982; van der Wilt et al., this volume). For positive as well as negative gravity orientation *Lymnaea* uses its statocysts. It is therefore conceivable that in *Lymnaea* efferent control of the statocysts plays a role in modulating the gravity orientation response (Janse, 1981; 1982). The experiments described in the present study are focussed on this issue. A more detailed account including sensory properties of the statocyst sensory cells will be published elsewhere.

Responses of statocyst sensory cells were recorded intracellularly upon gravity stimulation and upon stimulation of different types of sensory inputs in semi-intact preparations of *Lymnaea*. Moreover, responses were studied of the statocyst sensory cells to application of a number of drugs. Experiments were performed in a recording chamber with a volume of 8 ml. For the study of effects of drug applications, experiments were performed in a recording chamber with a volume of 1 ml .

In preparations consisting of the isolated CNS the morphology of the cells was studied by backfilling the axons of the statocyst sensory cells with HRP. This was done by pushing a broken microelectrode filled with HRP into the statocyst nerve. In this way in 5 preparations a total of 11 sensory cells were succesfully filled. Fig. 1 shows a diagram of the statocyst with a number of sensory cells. The statocyst is partly filled with statoconia, these are omitted in the Figure. The Figure shows that there are two types of cells: small (diameter about 30 µm) and large (diameter about 60 µm). This agrees with earlier findings of Geuze (1968). The Figure also shows that the cells were regularly shaped. Sensory cells with a regular shape have been described in *Hermissenda* (Detwiler and Alkon, 1973) and *Aplysia* (Janse, 1983).

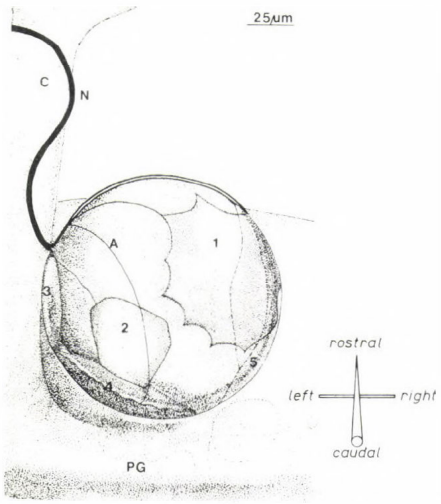

Fig. 1. Diagram of the left statocyst of the pond snail *Lymnaea stagnalis*. C: Pedo-pleural connective; N: Statocyst nerve; A: Axon of sensory cell; PG: Pedal ganglion; 1 - 5: Statocyst sensory cells.

Responses of statocyst sensory cells to tilt were studied in isolated CNS preparations. The recording chamber and microelectrode holder were placed on a platform (cast iron) which could be tilted maximally over 20°. A potentiometer monitored the position of the platform. Preparations were always positioned in such a way that that side of the statocyst containing the cells which were recorded from was the lower part during tilt. Cells were penetrated in different parts of the dorsal half of the statocyst. The resting membrane potential of the cells when the preparation was in horizontal position was between -50 and -75 mV.

In the horizontal position the activity of the cells varied between different preparations. Penetrations in the most dorsal part of the statocyst usually resulted in recordings with few or no action potentials and only small fluctuations of the membrane potential. Those near the equator resulted in recordings with a relatively high frequency of action potentials. Probably the activity level depends on the position of the sensory cell relative to the mass of statoconia (Janse, 1983). In addition the frequency and the amplitude of the fluctuations of the membrane potential in active cells were higher than in silent cells.

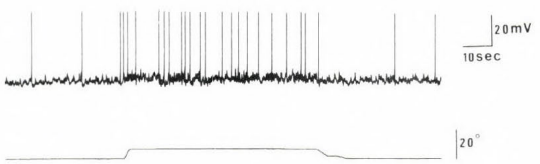

Fig. 2. Response of a statocyst sensory cell to tilt of the preparation. Upper trace intracellular recording. Lower trace position monitor.

During tilt the firing frequency of the cells and the frequency and amplitude of the membrane potential fluctuations increased (Fig. 2). Probably this increase is due to the statoconia which, during tilt of the preparation, move over the cell surface and collide with the cilia of the particular sensory cell (Grossman et al., 1979). The receptor potential probably consists of a summation of a number of such fluctuations (Stommel et al., 1980). At low frequencies the cells fired irregularly, at higher frequencies the firing was more regular. During a sustained tilt the cells adapted slowly (Fig. 2). Stimulus-response studies showed that the firing rate and the amplitude of the receptor potential were linearly related to the amplitude of tilt. Two types of cells were found which differed in sensitivity.

Efferent input to statocyst cells

In preparations consisting of parts of the body wall connected to the CNS, intracellular recordings were made of statocyst sensory cells during stimulation of peripheral sensory structures. Two types of preparations were used. 1. Preparations consisting of the CNS connected to the head-foot area and 2. Preparations consisting of the CNS connected to the mantle-lung area. In both types of preparations cells were penetrated near the equator of the statocyst, but in different areas. For the study of tactile synaptic input to statocyst sensory cells in a total of 15 preparations recordings of statocyst sensory cells could be obtained which permitted comparisons of responses upon stimulation of different parts of the skin. Mechanical stimulations of the skin were performed with a hand-driven glass probe with a

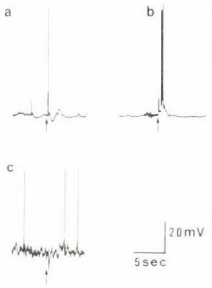

Fig. 3. Different types of responses upon tactile stimulation of the skin (arrows). a and b are of one preparation (see also text).

rounded tip (diameter: 0.5 mm). In both types of preparations responses could be obtained upon tactile stimulation of different parts of the skin (Fig.3). This indicates that the statocyst sensory cells receive tactile input from the entire body surface. Three types of responses were obtained (Fig. 3). a. A biphasic response consisting of a subthreshold depolarization or one or more action potentials followed by a hyperpolarization. b. An excitatory response resulting in a subthreshold depolarization or one or more action potentials. c. An inhibitory response resulting in hyperpolarizations of varying amplitudes. Stimulations of especially the lips, tentacles, mantle edge and the pneumostome were very effective in producing responses. The three types of responses could not always be recorded together in one preparation. The responses of the statocyst sensory cells upon tactile stimulation of the skin diminished considerably when the preparations were soaked in Ringer's solution with high [Mg^{2+}] and low [Ca^{2+}]. It is, therefore, concluded that the tactile input observed in the statocyst sensory cells is indeed of synaptic origin. Upon stimulation of head - foot - CNS preparations with light - on stimuli statocyst cells responded with a depolarization, which could also be induced by electrical stimulation of the nervus opticus. Obviously statocyst sensory cells receive also input from the eyes.

In preparations consisting of the CNS connected to the mantle with the mantle nerves, the responses of statocyst sensory cells were studied upon exposures of the mantle area to Ringer solution with high and low oxygen content and to air or N_2-gas. The lung cavity in these preparations was exposed by a transverse cut in the lung roof (see also Janse et al., 1985). The preparations were pinned down in a basin consisting of two compartments which could be separated with a perspex plate and grease (air and water tight) without severing the nerves between the CNS and the mantle. Each compartment had a volume of about 4 ml. The Ringer solution in the mantle compartment could thus separately be replaced by other Ringer solutions with different PO_2, by air or by N_2-gas (Janse et al., 1985). In general the statocyst sensory cells were extremely sensitive to small movements of the fluid surface. Replacing the Ringer solution with high PO_2 by solution with low PO_2 above the mantle area

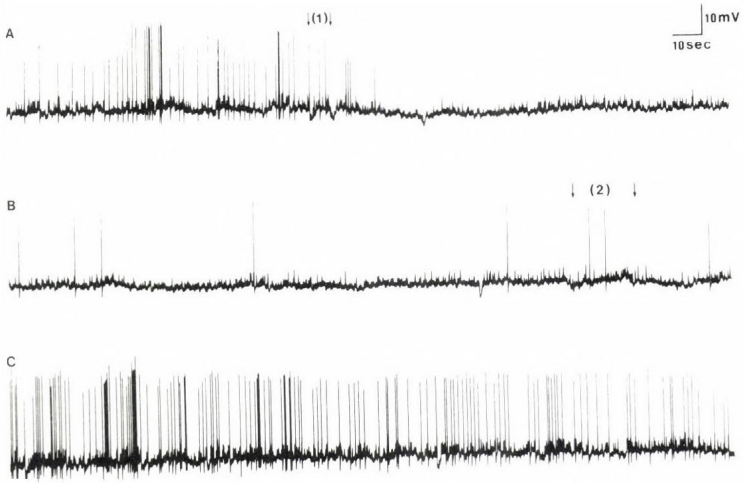

Fig. 4. Intracellular recording of a statocyst sensory cell during replacement of Ringer solution with high PO_2 with solution with low PO_2 (arrow 1) and vice versa (arrow 2). A - C are continuous.

produced a long lasting hyperpolarization. During the hyperpolarization the firing rate of the cells decreased. It was conspicuous that the membrane fluctuations stayed present (Fig. 4). Recovery of the membrane potential and firing frequency were obtained when the Ringer solution with low PO_2 was replaced again by solution with high PO_2. Similar results were obtained upon exposure of the mantle-lung area to N_2-gas or to air respectively. The responses upon PO_2 changes in Ringer or air were obtained in 6 preparations each.

Possible modulators of receptor activity

In isolated CNS the sensitivity of statocyst sensory cells was studied to a number of putative molluscan transmitters. Application of ACh and 5-HT (up to 10^{-3} M) did not produce responses. DA and NA (10^{-4} - 10^{-5} M) produced hyperpolarizations of about 5 - 10 mV of amplitude. Fig. 5 shows the response upon NA-application. The net response of the statocyst sensory cells upon a 10^{o} tilt decreased in the presence of NA or DA. The amplitude of the receptor potential upon tilt did not change. Fig. 6 shows the response of a statocyst cell upon tilt in the presence of NA. Similar results were obtained with DA.

To study the nature of receptors involved in the aminergic response, the following drugs were applied. Adrenaline (10^{-4} - 10^{-5} M) produced a hyperpolarizing response and a decrease of the firing rate. The β-blocker propanolol blocked the response upon NA whereas prazosin, an α-blocker, did not. Clonidine, an α-agonist, did not produce a hyperpolarizing response.

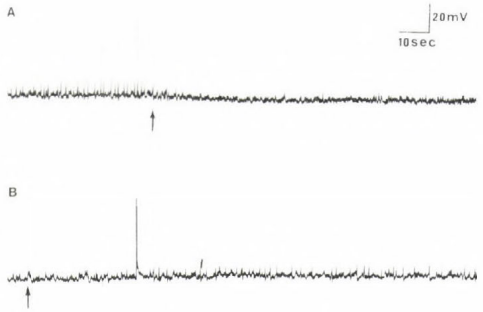

Fig. 5. Intracellular activity of a statocyst sensory cell during application of NA (10^{-4} M) (arrow in A) and after wash (arrow in B).

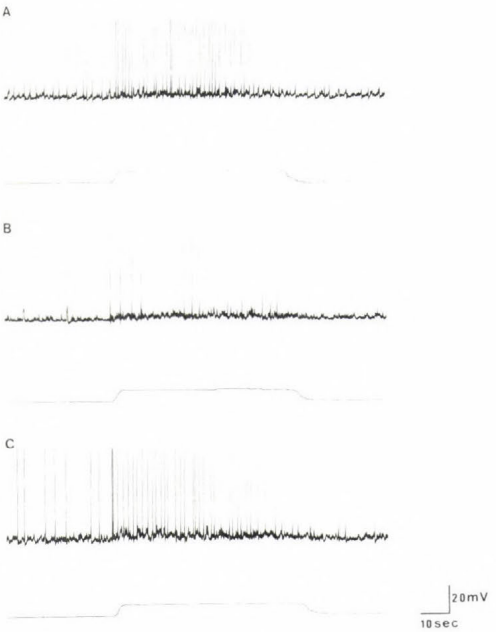

Fig. 6. Responses of a statocyst sensory cell in normal Ringer (A and C) and in Ringer with NA (10^{-5} M) (B). A and B are before and after NA application, respectively.

These experiments indicate that β- receptors are involved in the adrenergic hyperpolarizing response.

From the present results it appeared that the statocyst sensory cells receive efferent innervation from the entire skin area and from the eyes. Indications of efferent innervation of statocysts have also been obtained in other gastropods (in *Hermissenda*, Alkon et al., 1978 ; in *Aplysia*, Salánki and Jahan Parwar, 1985; and in *Arion*, *Limax* and *Helix*, Wolff, 1970),

in cephalopods (Budelman and Bonn, 1982; Williamson, 1986) and in vertebrates (Goldberg and Fernandez, 1980). The function of such an efferent innervation is in general related to an alteration of the afferent responses (Williamson, 1986). In *Lymnaea* efferent innervation of the statocyst sensory cells might be involved in the regulation of the sensitivity with which gravity orientation takes place. The observation that inhibitory input occurs on the statocyst sensory cells when the mantle and lung area are exposed to low PO_2 is in agreement with this idea.

As pointed out above efferent synaptic input may function in the regulation of the sensitivity of the statocyst sensory cells to tilt. This idea is supported by the observations made in the present study that application of noradrenaline during tilt of the preparation decreases the response of the statocyst sensory cells considerably. It is conceivable that noradrenergic transmision is of importance for the modulatory effects of the external PO_2 on the gravity orientation response. Indications that statocysts receive aminergic input have also been obtained in other molluscs (*Limax*: Osborne and Cotrell, 1971; cephalopods: Budelmann and Bonn, 1982). Noradrenaline has been shown to be present in gastropod nervous systems by Franchini et al. (1985). In *Lymnaea* still other transmitters or modulators are probably also involved in efferent control of the statocyst cells because the cells also receive excitatory synaptic input from the skin and the eyes.

It is not known how the information on the external PO_2 reaches the statocyst cells. It is however known that exposure of the mantle area to different PO_2 affects the activity in peripheral nerves (Janse et al., 1985). It is also known that a great number of identified cells respond to changes in the PO_2 above the mantle - lung area (Janse et al., 1985). Some of the neurons are peptidergic and are involved in respiratory behaviour. Especially two giant ACTH-like peptide containing cells seem to be important. On the basis of their morphology these neurons can be inferred to have wide spreading effects (van der Wilt et al., this volume). It is conceivable that some of these peptidergic neurons also are involved in the modulation of gravity orientation. Apart from interactions of gravity orientation behaviour with respiratory behaviour there are also indications that interactions exist between orientation and egg - laying (van der Wilt, unpublished). Such interactions may also involve peptidergic modulation. It might therefore be of interest to study in future experiments the sensitivity of statocyst cells or their follower cells in the CNS to relevant peptides.

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DISCUSSION

ALKON, D.L.: Have you entirely ruled out the possibility that the sensory hair cells themselves are sensitive to changes in O_2 tension? I believe there is some evidence of such sensitivity in other systems.

JANSE, C.: Yes, we applied the changes in O_2 only in the compartment containing the mantle. In the intact animal you might indeed expect changes in O_2 when the animal is kept on O_2-low conditions.

BLANKENSHIP, J.E.: 1. Was CO_2 and/or pH controlled in bath over lung/mantle? Is this a true O_2 receptor?

2. What is the evidence that noradrenaline is a transmitter in gastropods?

JANSE, C.: 1. Yes. Moreover, responses to changes in external PO_2 were also obtained upon exposure of the mantle area to air with different PO_2.

BRÄUNIG, P.: You showed the suppression of statocyst cells by low PO_2 in the periphery. Does that imply that the snail cannot use its statocyst when orienting towards the water surface?

JANSE, C.: Actually we expected to find also statocyst cells which are excited by exposure of the mantle/lung area to low PO_2. That we did not find such cells might be due to the fact that we could not make recordings from all statocyst cells.

MOFFETT, S.: Have you examined the effect of maintaining oxygen levels constant and varying the carbon dioxide levels?

JANSE, C.: No we did not, but van der Wilt has indication that CO_2 levels influence orientation behaviour.

SALÁNKI, J.: 1. When you applied the drugs to the preparate, did it affect the statocyst directly or through synaptic contacts?

2. Did you try serotonin, and if so, what was the effect?

JANSE, C.: 1. The drugs were applied to the isolated CNS. It is therefore possible that the effects are mediated by central neurons.

2. We did apply serotonin but it produced no effects.

SYED, N.I.: In your abstract you have mentioned the involvement of an extensive network acting on your system, but you have not shown us any example - were you referring to van der Wilt's paper?

JANSE, C.: I was referring to the neurons which receive information from peripheral O_2-sensitive structures. Part of these neurons are involved in respiratory behaviour, and are described in Janse et al. (1985).

Symposia Biologica Hungarica 36 (1988)

CONTROL OF OVIDUCT MOVEMENT IN PERIPLANETA AMERICANA

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ABSTRACT

The oviducts of the cockroach Periplaneta americana are innervated by a pair of nerves coming from the terminal ganglion, the so-called nerves VII C (nomenclature after Roeder et al. 1960). The corresponding somata (up to 25) form three distinct groups: 12 somata in the anterior region of the dorsal midline (DUM-neurons?), up to 9 somata laterally (8 ipsi-, 1 contralaterally) in the neighbourhood of the origin of nerve VII C, and 4 somata in the ventral midline of the terminal ganglion (VUM-neurons?).

Octopamine enhances the frequency and amplitude of the spontaneous phasic contractions of the oviduct in lower, and reduces them in higher concentrations. Norepinephrine < serotonin < proctolin induce tonic contractions of the oviduct combined with alterations of the frequency and amplitude of the spontaneous phasic contractions. Consecutive addition of octopamine (10^{-6} M) and proctolin or serotonin potentiates the enhancement effect of proctolin or serotonin upon the frequency of the phasic contraction and at the same time reduces the induced tonic contractions. The octopamine effect is sensitive to phentolamine, but not to propranolol. The role of octopamine as a neuromodulator at the oviduct muscle is discussed.

INTRODUCTION

Recently, Orchard and Lange (1987) demonstrated that both octopamine and proctolin are present in cockroach oviducts and are released in a calcium-dependent manner following depolarization with high potassium saline. It is not known until now whether octopamine has an effect on cockroach oviduct muscle. Proctolin has been shown by Holman and Cook (1985) to induce an increase in muscle tonus and an increase in amplitude and frequency of phasic contractions in Leucophaea maderae. It may have a similar effect in Periplaneta. In the oviduct muscle of Locusta migratoria octopamine is believed to act as a neuromodulator whereas proctolin may be an excitatory co-transmitter with a "classical" transmitter such as glutamate (Orchard and Lange 1985, 1986; Lange et al. 1986).

Innervation of the cockroach oviduct muscle

The oviducts of Periplaneta are innervated exclusively via the nerve VII C (nomenclature after Roeder et al. 1960) originating from the terminal ganglion. This nerve consists of 25 axons. In accordance with this number of fibres 25 perikarya could be stained by retrograde cobalt sulfide iontophoresis in the terminal ganglion. These somata form three distinct groups in the anterior region of the ganglion (Fig. 1).

Immunocytochemically, one cell of group 3 could be identified as proctolin-containing (Agricola et al. 1985, Stoya et al., in press). At the dorsal surface of the oviduct muscles a thin network of fibres with proctolin-like and serotonin-like immunoreactivity could be demonstrated. Whereas serotonin-like immunoreactivity was more restricted to the common oviduct, proctolin-like immunoreactivity was distributed over both the lateral and the common oviduct (Stoya et al., in press).

Effect of octopamine and other catecholamines on the oviduct

Addition of octopamine to the bath fluid produced a change in the amplitude and frequency of spontaneous phasic contrac-

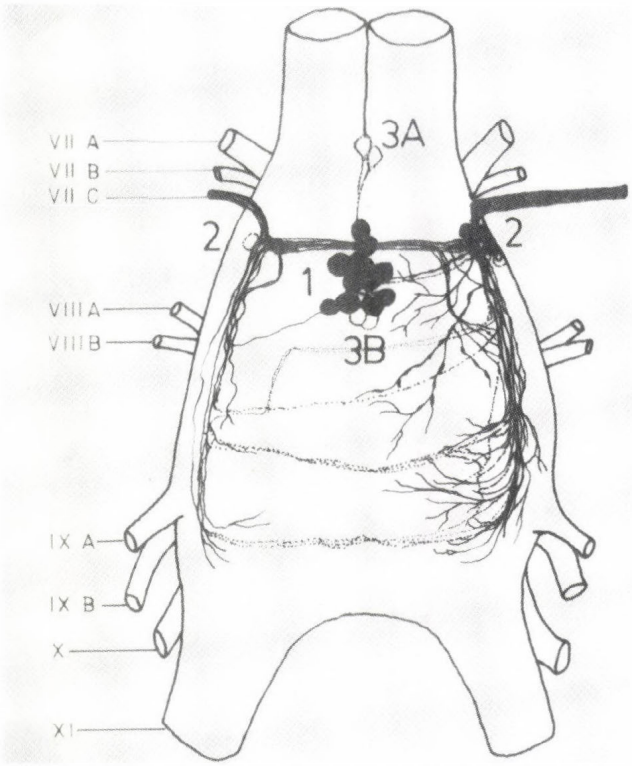

Fig. 1. The 6th abdominal ganglion (dorsal view) after unilateral retrograde $CoCl_2$-iontophoresis via oviduct innervating nerve VII C. The ventral elements are dotted. 1, 2, 3A, 3B - oviduct innervating neurons of groups 1, 2, 3A and 3B. Group 1: twelve unpaired somata along the dorsal midline of the ganglion (DUM-neurons). Group 2: up to nine somata at the edge of the ganglion (eight ipsi- and one contralateral) in the neighbourhood of the origin of nerve VII C. Group 3: four unpaired somata along the ventral midline of the ganglion (VUM-neurons).

tions of isolated oviduct. In lower concentrations (10^{-9} or 10^{-8} mol x l^{-1}) octopamine enhanced the frequency and amplitude of the phasic contractions immediately after addition, whereas in higher concentrations (10^{-6} or 10^{-5} mol x l^{-1}) it reduced both parameters temporarily (Fig. 2). The muscle tonus is only slightly reduced. A mean concentration of 10^{-7} mol x l^{-1} had no significant effect on the musculature. The dose-response curve relating the frequency of phasic contraction of oviduct

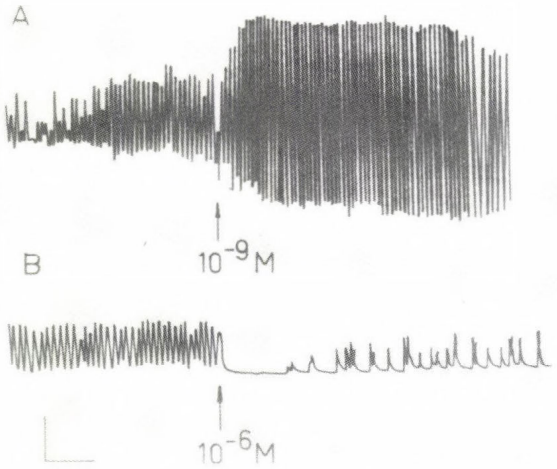

Fig. 2. Effect of octopamine on the isolated oviduct muscle. (A) Elevation of the frequency and amplitude of the spontaneous phasic contractions after application of 10^{-9} mol/l octopamine. (B) 10^{-6} mol/l octopamine temporarily reduced the frequency and amplitude of the spontaneous phasic contractions. The muscle tonus is slightly reduced. Horizontal time mark = 1 min.

one minute after addition to the octopamine concentration applied is shown in Fig. 3.

No other catecholamine tested (tyramine, dopamine, adrenaline: 10^{-6} mol x l^{-1}) was as active as octopamine. In contrast to octopamine, noradrenaline (10^{-6} mol x l^{-1}) increased the frequency of spontaneous phasic contractions.

Effects of serotonin and proctolin on the oviduct

The indolealkylamine serotonin (5-HT) as well as the neuropeptide proctolin changed three parameters of the activity of the isolated oviduct dose-dependently (Figs 4 and 5):

1. the muscle tonus
2. the amplitude and
3. the frequency of spontaneous phasic contractions

All these effects were reversible. They could be washed out with Ringer solution. The threshold for excitation was 10^{-11} mol x l^{-1} x l^{-1} in the case of proctolin and 10^{-9} - 10^{-8} mol x l^{-1} in the case of serotonin. The maximum shortening was

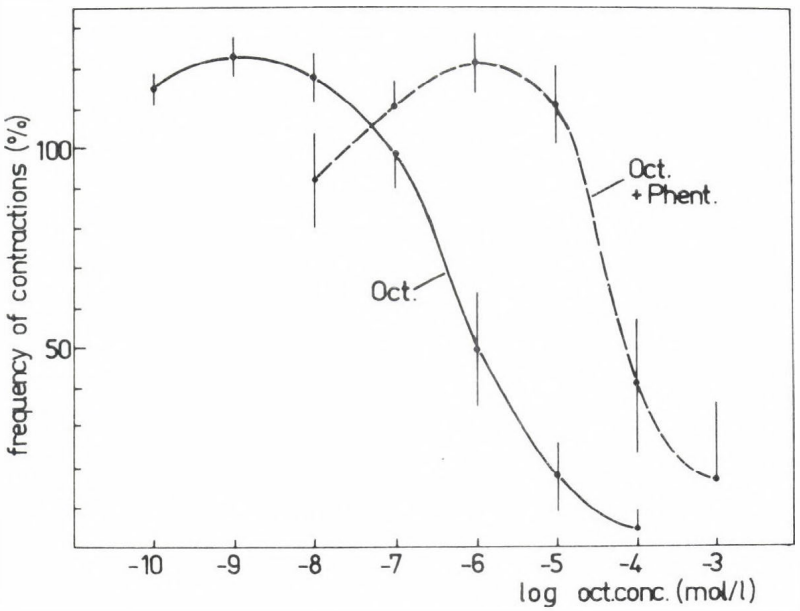

Fig. 3. Dose-response curve of the effect of octopamine upon the frequency of the spontaneous phasic contractions of the isolated oviduct muscle one minute after application. In the presence of the adrenergic blocker phentolamine (Phent.) (2.3×10^{-6} mol/l) the dose-response curve of octopamine was shifted to the right. Points represent mean ± SEM, n = 7-8.

reached with 10^{-6} mol x l^{-1} proctolin and was much more intense than with serotonin.

Of special interest is the interaction of octopamine with serotonin or proctolin at the oviduct. As already mentioned, octopamine 10^{-6} mol x l^{-1} inhibited the frequency of spontaneous phasic contractions of cockroach oviduct whereas proctolin stimulated it. This stimulating effect of proctolin could be enhanced by octopamine, when it was applied 30 s before. At the same time the proctolin induced tonic contraction of the oviduct was reduced in the presence of octopamine (Fig. 6). The dose-response curve was displaced to the right in a parallel manner (Fig. 7).

Octopamine 10^{-6} mol x l^{-1} had the same effects on the frequency of spontaneous phasic contractions and the tonic con-

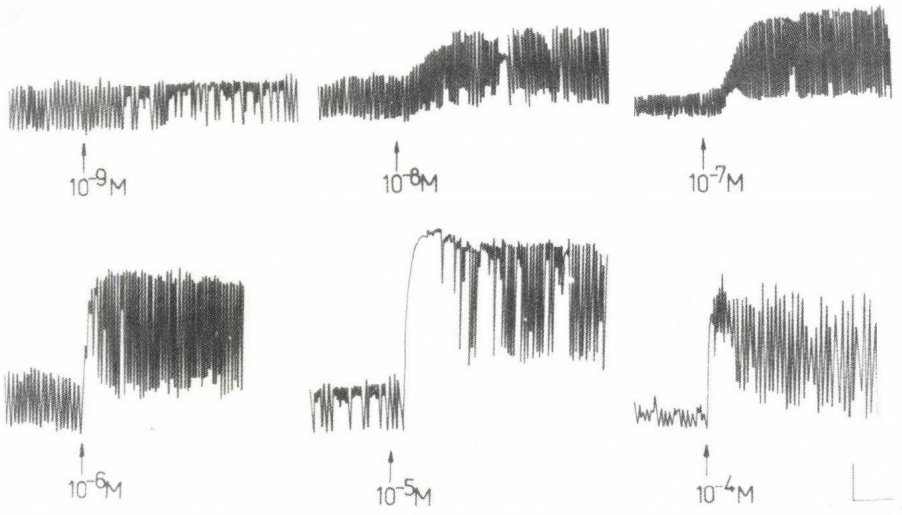

Fig. 4. Effect of increasing concentrations of serotonin (5-HT) on the isolated oviduct. Between the tests the preparation was rinsed with saline solution. Each response was reversible. Horizontal time mark = 1 min. Verticular calibration = 130 µm tissue displacement.

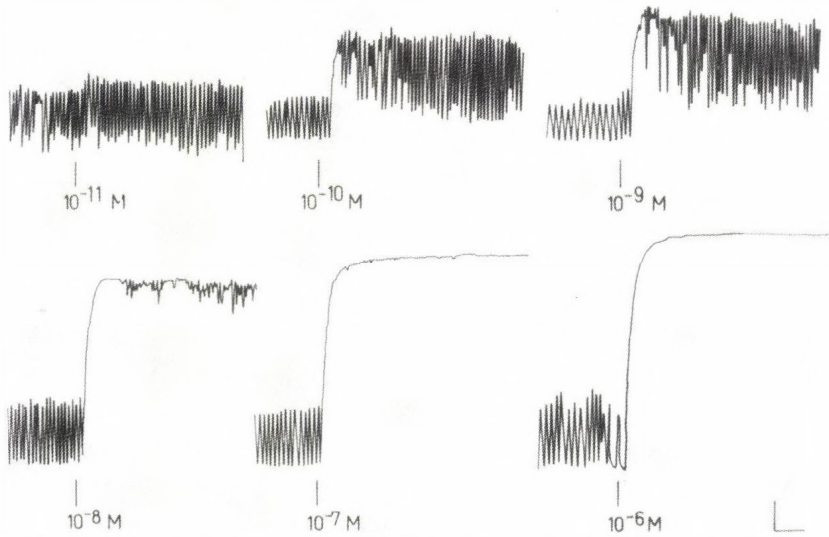

Fig. 5. Effect of increasing concentrations of proctolin on the isolated oviduct. Between the tests the preparation was rinsed with saline solution. Each response was reversible. Horizontal time mark = 1 min. Vertical calibration = 130 µm tissue displacement.

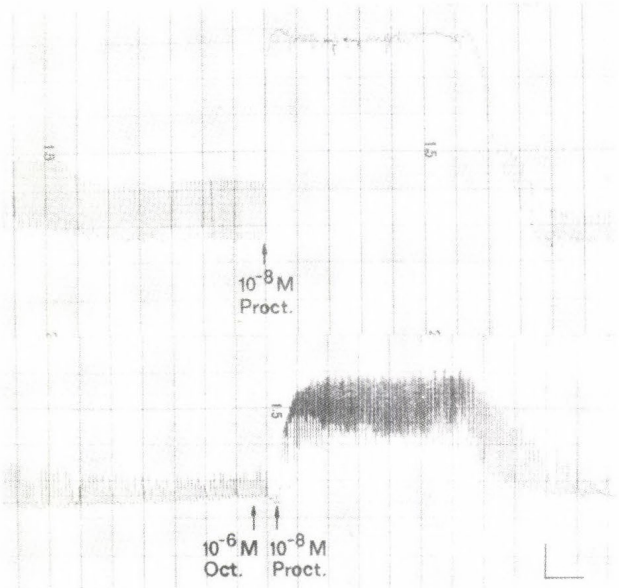

Fig. 6. Presence of octopamine (10^{-6} mol/l) enhances the frequency of spontaneous phasic contractions and reduces the tonic contraction of the oviduct muscle induced by proctolin (10^{-8} mol/l).

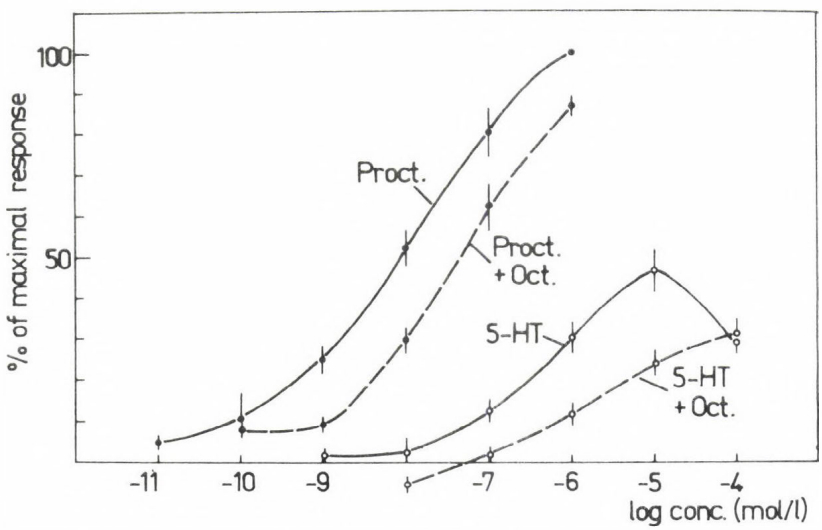

Fig. 7. Dose-response curves for proctolin (●) and 5-HT (o) in the absence or presence of 10^{-6} mol/l octopamine (Oct.) (added 30 s before application of the agent). In both cases the dose-response curves were displaced to the right in a parallel manner. The response is expressed as a percentage of maximum tension obtained with proctolin. Points represent mean ± SEM, n = 8-10.

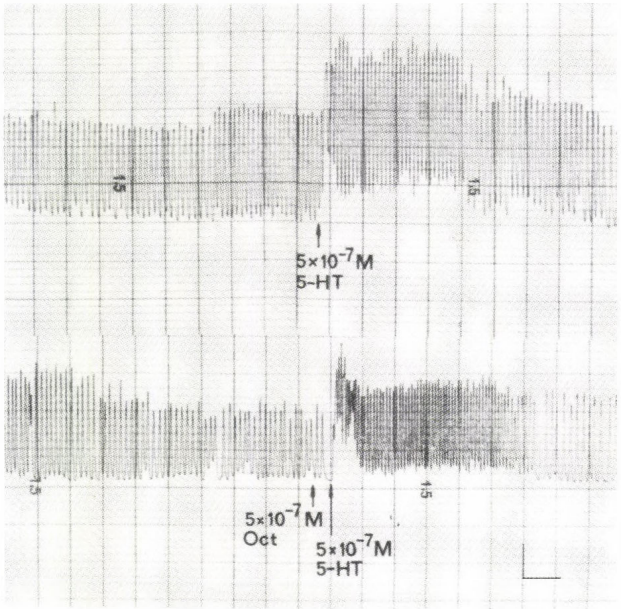

Fig. 8. Presence of octopamine (5×10^{-7} mol/l) enhances the frequency of the spontaneous phasic contractions and reduces the tonic contraction of the isolated oviduct muscle induced by serotonon (5-HT) (5×10^{-7} mol/l).

traction of oviduct muscle induced by serotonin (Fig. 8, see also Fig. 7).

The influences of adrenergic blockers

In presence of the α-adrenergic blocker phentolamine (2.3×10^{-6} mol $\times$ l^{-1}) the dose-response curve of octopamine with respect to the frequency of the spontaneous phasic contractions was displaced to the right without changing its characteristic course (see Fig. 3). In contrast, the increasing effect of proctolin (10^{-9} mol $\times$ l^{-1}) or serotonin (10^{-6} mol $\times$ l^{-1}) on the frequency of the spontaneous phasic contractions remained unaffected in the presence of phentolamine.

The α-adrenergic blocker propranolol had no influence upon the responses of octopamine, proctolin or serotonin.

CONCLUSION

In conclusion, we can state that the oviducts of Periplaneta americana are exclusively innervated by up to 25 neurons of the terminal ganglion via so-called nerves VII C. Some of these neurons are octopaminergic, others proctolinergic (Agricola et al. 1985, Sobek et al. 1986, Stoya et al., in press).

The cockroach oviducts are very sensitive to proctolin. The threshold for excitation was about 10^{-11} mol x l^{-1}. Octopamine enhanced the frequency and amplitude of the spontaneous phasic contractions of the oviduct in lower, and reduced them in higher concentrations. It is supposed that proctolin acts as an excitatory neurotransmitter at the cockroach oviducts whereas octopamine plays the role of a neuromodulator. This is, apparently, also true for locusts (Orchard and Lange 1985, 1986). In our opinion, octopamine and proctolin act synergically: Octopamine changes the responsiveness of the oviduct to proctolin favouring high frequencies of the spontaneous phasic contractions and thus supporting the transport of mature eggs within the oviducts.

The role of serotonin on the oviduct remains unclear. In comparison to proctolin serotonin was 1000-fold less effective at the oviduct. From a comparative point of view it is interesting that the locust oviduct is insensitive to serotonin (Lange and Orchard 1984). In our laboratory Eckert et al. (unpublished) immunocytochemically demonstrated serotonin-like immunoreactivity in cockroach oviducts.

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DISCUSSION

BLANKENSHIP, J.E.: Which neurons are octopaminergic?

PENZLIN, H.: In the system I talked about in my lecture the main source for octopamine may be the DUM-neurons which are in connection with the oviduct via nerve VII C.

BRÄUNIG, P.: I am intrigued by the number of DUM-neurons you found in this system. Do you think there is a kind of peripheral - area - fractionation between them?

PENZLIN, H.: Yes, there is. Eight somata of group 1 send their axons to the sphincter muscle, whereas only two DUM-neurons innervate the lateral oviduct.

ELEKES, K.: Did you observe any differences in OA-response when applying in males and females, respectively?

PENZLIN, H.: We had only females (oviduct) in our experiments.

FLOREY, E.: Did you investigate the action of acetylcholine in the oviduct preparation?

PENZLIN, H.: No, not yet.

MOFFETT, S.: Has it been possible to measure the blood levels of these transmitters in ovipositing cockroaches?

PENZLIN, H.: In respect to proctolin it is impossible because the enzymatic destruction of this neuropeptide in the haemolymph occurs very fast. The octopamine level in the haemolymph has been measured by Evans (1985) in locust (10 mg/g tissue).

IONIC CHANNELS AND INTRACELLULAR MECHANISMS

Symposia Biologica Hungarica 36 (1988)

THE IONIC BASIS OF THE MULTIFORM Ca-DEPENDENT SPIKES IN MOLLUSCAN NERVE CELLS

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Central nervous system neurons are endowed with a great variety of different membrane ionic channels. This wide diversity accounts for their integrative properties and their specific signaling characteristics. It has been recognized for several years that Ca ions play a central role in the spike patterning of central neurons. Ca-dependent electrogenesis differs markedly from Na-dependent, Hodgkin-Huxley electrogenesis, which is of a stereotyped nature. By contrast, Ca-dependent signals include a wide variety ranging from graded responsiveness to multiple spiking and epileptiform discharges. This diversity is partly related to the properties of Ca current which is characterized by long-lasting, persistent activation and by slowly developing inactivating processes. It is also related to the recurrent action of penetrating Ca ions on the membrane.

In the resting neuron, cytosolic calcium is in the range of 100nM. During activity Ca values may rise substantially, particularly in the cytoplasmic domain immediately adjacent to Ca channels (Chad and Eckert, 1984). The elevated Ca may in turn regulate a number of Ca-sensitive ionic channels (Fig.1). This includes the Ca channel itself and several types of Ca-activated channels. A large number of passive and active processes tend to limit the Ca diffusion and to prevent excessive cytotoxic intracellular Ca accumulation: Ca ions are buffered by Ca-binding proteins; membranes and organelles (endoplasmic reticulum, nucleus, vesicular structures) participate in Ca sequestration and Ca ions are actively pumped out from the cytoplasm through the plasma membrane and by mitochondria.

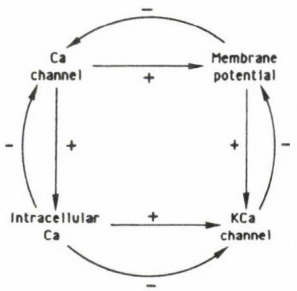

Fig. 1. Sequential ionic and electrical events following a Ca entry in nerve cells endowed with Ca and Ca-dependent K channels.

Intracellular Ca injections into Helix neurons induce the activation of several specific ionic currents, particularly K currents (Meech, 1978). These Ca-activated K currents have been thought to be involved in long-lasting, post discharge events as well as in fast repolarizing processes within the spike (reviewed by Schwartz and Passow, 1983; Petersen and Maruyama, 1984). In addition, elevated cytosolic Ca concentration may decrease outward K currents (Heyer and Lux, 1976). These opposite effects of penetrating Ca ions onto Ca and K channels result in transient imbalances which favor either cell repolarization or excessive discharges.

In view of all the above properties of Ca-dependent electrogenesis it is not surprising that great diversity of signal patterning is observed in cells governed by Ca currents. We have analysed several types of Helix nerve cells in which Ca is the main carrier of the inward current producing spiking activity. Since these cells do not differ as regards the nature of the Ca channels we have focused our study on the mechanisms involved in spike repolarization, i.e. on the repolarizing voltage-gated and Ca-gated K currents.

Two cell groups were selected. They both have a high density of Ca channels and relatively few or no Na channels. Under repeated stimulations they display, however, two opposite behaviours. The first group, identified as U cells by Lux and Hofmeier (1982), shows spike attenuation and fast adaptation under low frequency (1-3 spikes/s) firing (Fig.2A1). By contrast, in the second group, styled P or plateauing cells, the spike enlarges considerably during a burst (Gola, 1985); at a moderate spike frequency (around 5Hz) the

repolarization aborts and the spike converts to a long-lasting (several seconds) depolarized plateau (Fig.2B1). This plateau resembles the paroxysmal depolarization described in cortical neurons of epileptic foci or those induced by convulsants.

The main ionic conductances present in both cell types have been determined using pharmacological and ionic dissection of the inward and outward currents.

Fast adapting U cells lack Na and V-gated K currents (Lux and Hofmeier, 1982). The large (100-115mV) spike is produced by the successive opening of Ca and Ca-activated K channels, as shown in the diagram in Fig. 1. The blocking effect of KCa channels induced by elevated calcium does not exist in U cells,i.e. the KCa current persists in long depolarizations (Fig.2A2).

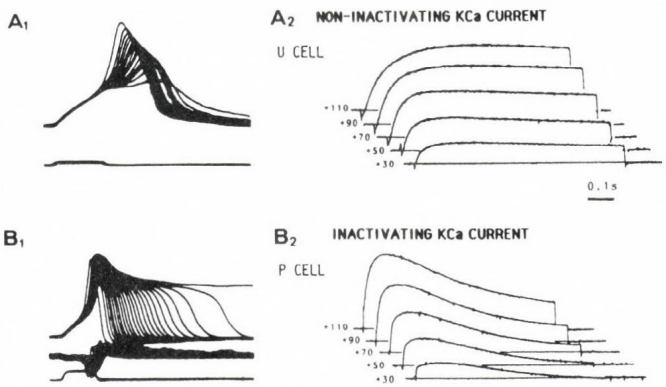

Fig. 2. Contrasting spike changes in Ca-dependent U and P neurons. Low frequency stimulation either produces spike attenuation (U cell) or results in long-lasting plateau (P cell). Right row shows families of Ca-dependent K currents produced in response to increasing depolarizations in both cells.

Plateauing neurons are mainly endowed with Ca channels and with V-gated KV and Ca-activated KCa channels (Gola, 1985). Both K currents display considerable relaxation in prolonged depolarization (Fig.2B2): inactivation of KV channels is a voltage-dependent process; that of KCa channels is produced by intracellular calcium (Gola et al., 1986a) (see in Fig. 1 the arrows indicating delayed blockade of KCa channels by Ca).

The properties of the KCa current in U cells have been described by Lux and Hofmeier (1982). This current has two important peculiarities: 1) its onset in response to step depolarizations becomes very slow at large positive potentials (Fig.3A), which contrasts with the acceleration exhibited by most V-gated K channels; 2) a previous Ca entry accelerates the activation rate in a voltage-independent manner (Fig.3B). The facilitation exerted by a brief Ca entry shades off within 1s, reflecting the relatively fast return of the intracellular $[Ca]_i$ to its rest level.

These experiments provide strong indications that several steps, controlled by potential and Ca, are involved in KCa channel opening. It has been suggested that Ca may have a permissive effect on the channel opening: a rise in $[Ca]_i$ might be necessary for the opening to occur and the open state might then be maintained by cell depolarization. Fast pressure injection of Ca blockers, performed just after KCa current activation by a step depolarization, induced a current relaxation. Additional depolarizations failed to restore the current. This experiment clearly shows that a permanent Ca inflow is necessary to maintain the KCa channel in the open state.

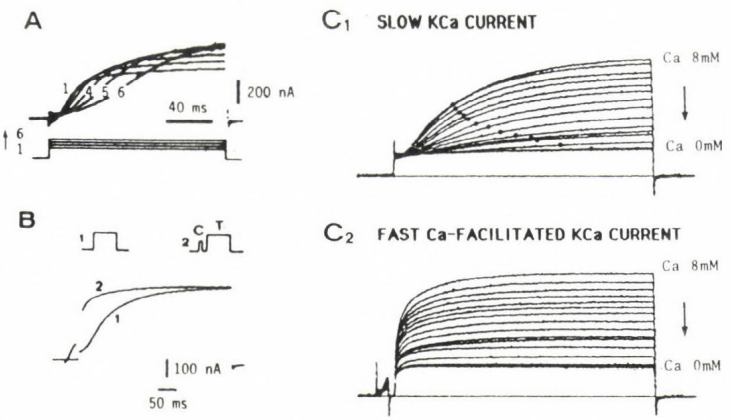

Fig. 3. Slow and fast opening modes of KCa channels. A: family of slow KCa currents produced in response to increasing depolarizations. B: slow (1) and fast (2) KCa current: the fast onset results from the facilitating effect by Ca ions entering the cell during the C pulse. C: effect of bath Ca concentrations on the slow (C_1) and fast (C_2) KCa currents (half-activation indicated by dots).

Changing the Ca content of the perfusing saline altered both the steady-state KCa current and its activation rate (Fig.3C1): both parameters were directly related to the extracellular Ca content, i.e. to the flow of penetrating Ca ions. Under the same conditions, the fast activation rate or facilitated opening mode was not dependent on the Ca inflow (Fig.3C2). These observations strongly suggest that the channel opening occurs after successive steps controlled either by Ca or by voltage. The sigmoid activation rate of the KCa current and its changes with calcium indicate that at least two steps are Ca-dependent or that at least two Ca ions bind to the channel. In the Ca-bound state, channels would be ready to be opened by membrane depolarization and this opening would be a fast process.

In conclusion, the Ca dependency of the KCa current amplitude and activation rate makes this current particularly suited to counteracting the depolarizing effect of Ca currents; a large Ca entry will produce a fast, large KCa current able to rapidly repolarize the cell and to prevent the expression of the sustained depolarizing Ca current.

Information concerning the voltage-controlled transition from the Ca-bound state to the open state has been hard to obtain in whole-cell voltage clamp experiments since changing the voltage will modify the Ca inflow. To solve this problem we performed patch clamp experiments in the cell-attached configuration.

INSIGHTS AT THE MICROSCOPIC LEVEL

A great deal of information on single Ca-activated K channels in muscle and secretory tissues is available whereas very few experiments have been performed on similar channels in neurons. A large-conductance channel (200pS) has been described in cultured rat sympathetic neurons (Smart, 1987). The KCa channel in molluscs has a far smaller unitary conductance (19pS in Helix, Lux et al., 1981; 39pS in Aplysia, Hermann and Erxleben, 1987). In order to unambiguously identify unitary currents as flowing through KCa channels, experiments were performed in U cells (almost devoid of

V-gated K channel) under various experimental situations. The selection criteria were: 1) the unitary currents must reverse direction at the equilibrium potential for K ions (changed from -60 to OmV by raising the K content of the patch electrode); 2) they must be blocked by intracellularly injected Ca chelators (from an additional intracellular EGTA-filled microelectrode); 3) they must display the same voltage-dependency as the whole-cell KCa current (decreased opening probability at large patch depolarizations) and 4) they must reproduce the facilitation phenomenon in response to a large Ca entry. Using these criteria, we characterized the KCa channel as having a 32pS unitary conductance when the patch electrode was filled with physiological saline. The conductance increased to 50pS with isotonic K or free-Ca salines. The sensitivity of the channel conductance to extracellular Ca and K concentrations may account for the large variations reported by investigators (Ewald et al., 1985).

Fig. 4a shows a typical recording of KCa channel currents in response to a step depolarization of the patch membrane. We note that 1) no channel opening occurs until the patch is depolarized; 2) this patch contains at least 3 channels and 3) the probability of opening decreases progressively as the patch is held at positive potentials.

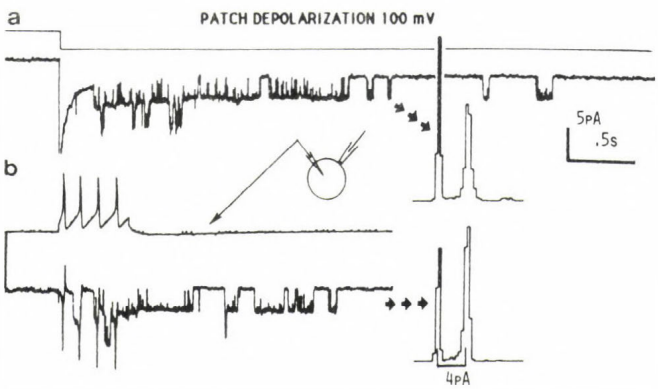

Fig. 4. Single KCa channels. a: transient openings of KCa channels in response to step depolarization of the membrane patch. b: burst of channel opening induced in the quiescent depolarized patch by action potentials triggered by whole cell stimulation.

Point 3 seems to indicate that an inactivating process is operating in the depolarized patch, as opposed to the non-inactivating behaviour of the whole-cell KCa current. Actually, inducing a large Ca entry through the membrane surrounding the patch (performed with an intracellular electrode) produced a transient burst of channel openings in the quiescent depolarized patch (Fig. 4b). This process could be repeated regularly and the response still persisted in spite of the accumulation of Ca within the cell. Therefore, we concluded that there is no inactivating process (either induced by depolarization or Ca-dependent) acting on the KCa channels in U cells. The progressive decrease in opening probability merely results from the relaxation of the Ca current induced by the patch depolarization. This experiment confirms the conclusion issued from whole-cell voltage clamp experiments concerning the Ca requirements for maintained activation of the KCa current.

Point 1 leads to two main conclusions. First, the channel opening probability must be strongly voltage-dependent so that no channel opening can occur at the resting membrane potential even when large Ca currents are produced in the non-clamped membrane. Secondly, the indispensable Ca inflow that sustains the initial burst must be produced by the patch depolarization, i.e. the patch must also contain Ca channels, the functioning of which is masked by the large, overlapping K currents. Indeed, with a patch electrode filled with Ca-free saline, the initial burst was lacking, whereas openings were still observed in the depolarized patch in response to Ca inflow through the non-clamped membrane. These data, together with the above point 2, suggest that KCa channels are gathered in small clusters surrounding Ca channels. Generally the patch contains either no KCa channel or groups of at least 3 channels. We also observed spontaneous bursts of channel openings in quiescent depolarized patches. The bursts lasted about 1s, they occurred very scarcely and they were separated by long periods of closure. They were produced by the almost simultaneous opening of 3 to 7 channels. The bursts are difficult to interpret in terms of transient openings from inactivated states, since such a process would affect individual channels in a random fashion. It is more probable that the bursts may occur in response to the transient opening of one Ca channel within

the patch. The cytoplasmic restricted domain in which the entering Ca ions are sequestered would thus include 3 to 7 KCa channels.

The experimental procedure shown in Fig. 4b was used to assess the voltage-dependency of the opening probability. For this purpose, the patch electrode was filled with Ca-free saline and the patch potential was stepped to increasing levels. No opening occurred in response to the step depolarization owing to the absence of Ca on the extracellular side of the membrane patch. Ca was then allowed to enter the cell through the non-clamped membrane during a single spike triggered by an intracellular electrode. With this procedure, it was possible to determine the change in channel opening probability in response to a given Ca entry and at various membrane potentials. The results showed that detectable open events required 25 to 30mV depolarization (see also Hermann and Erxleben, 1987). As the patch was progressively depolarized, opening occurred more frequently and the number of simultaneous openings increased. Repolarization performed during a burst of synchronous openings immediately closed the channels. These findings are in agreement with the existence of a sharp control of opening probability exerted by cell polarization.

Conclusion

The pronounced voltage sensitivity and the rapid relaxation kinetics of the KCa current present in U cells suggest that this current may play some role in the repolarization of the action potential. They also suggest that the KCa current may not contribute substantially to the potential trajectory between spikes and following active periods nor to the hyperpolarizations induced by Ca injections in resting neurons (Meech, 1978). Since such long-lasting hyperpolarizing events are undoubtedly related to the activation of K currents by calcium, our data promotes the hypothesis that several distinct Ca activated K channels exsist in molluscan neurons: fast activated channels governing the spike configuration and slowly activated channels involved in firing modulation. Similar functional distinctions between two Ca-dependent processes have been reported in bull-frog sympathetic neurons (Pennefather et al., 1985) and rat hippocampal neurons (Lancaster and Nicoll, 1987).

In order to assess the role of KCa current in cell firing characteristics, we have developed a method with which it is possible to detect specific ionic currents during cell activity. The method, adapted from an idea by Llinas et al. (1982) makes use of spike-like voltage clamp pulses. In brief, the spontaneous spike from a freely firing cell was collected and stored in a digitized form. The recorded spike was then applied to the command input of the voltage-clamp feed-back device. When spike recording and injection were performed under the same conditions, the feed-back amplifier delivered no net current. On the contrary, when one channel type was specifically blocked, the corresponding current had to be generated by the voltage-clamp circuit (Gola et al., 1986b). By successively blocking the various channels present in the cell under investigation, the contribution of the corresponding currents to the spike wave-form can be resolved.

This method has been applied to the Ca-dependent U cells. First, the KCa current contribution to the spike was extracted by blocking the channels with either extracellularly applied TEA or intracellularly injected EGTA. The Ca current time course was then measured by adding Ca channel blockers to the TEA-treated or EGTA-injected cell. The results are given in Fig.5A.

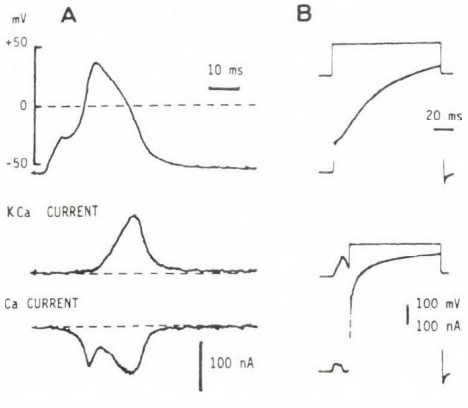

Fig. 5. KCa current involvement in spike repolarization. A: Ca and KCa currents underlying the Ca-dependent spike in U cell. B: spikes convert the slow KCa current (upper recording) into the fast activated mode (lower recording).

1) Ca current: its activation started during or just after the current pulse used to trigger the spike. It repeatedly showed two peaks, corresponding to the uppermost depolarizing phase and the shoulder of the repolarizing phase, respectively. Ca currents in molluscs have relatively slow opening kinetics. The large delayed Ca current peak reveals that the Ca channels opened progressively. The transient decrease concomitant with the spike overshoot must correspond to the reduction in driving force acting on Ca ions while recruitment of opened Ca channels was still in progress. This reduction must result both from the depolarization and from the reduction in Ca reversal potential after the first Ca entry. The delayed Ca peak was transiently produced both by maintained depolarization, allowing Ca channel recruitment, and by increased driving force during slow spike repolarization.

2) KCa current: the KCa current change was faster than expected from voltage-clamp data. None of the modeling studies incorporating Ca-activated outward currents have anticipated such fast behavior, but predict slow, cumulative increases due to intracellular Ca accumulation during spike trains. In U cells, KCa channels started to open as the spike overshot and the KCa current peaked almost simultaneously with the Ca current, i.e. at half-repolarization. This relatively fast increase can be accounted for by the opening dynamics of KCa channels, which have both slow and fast kinetics. The fast opening mode was observed when voltage-clamp pulses were applied just after a large Ca entry. The first surge of Ca ions during the spike depolarization may have converted a large proportion of KCa channels into the fast opening mode. This hypothesis has been tested using the experimental arrangement shown in Fig.5B. In response to a large depolarizing pulse, the KCa current displays its characteristic slow sigmoidal increase. When the step was performed on the falling phase of an isolated spike (by electronically switching the electronic device to the voltage-clamp conditions), the KCa current then had the fast dynamics attributed to the facilitation by previous Ca entries (see Fig.3A). Therefore, due to the first Ca surge during the spike, most KCa channels are in the closed Ca-bound state and ready to be opened by cell depolarization.

Although voltage-clamp experiments have characteristically led to the KCa current being described as a slow system, it now emerges that in freely firing cells, this current operates mainly in the fast facilitated mode and contitutes a powerful spike repolarizing mechanism. Evidence is now gathering that a similar Ca-dependent K current repolarizes action potentials in vertebrate neurons (Adams et al., 1982; Pennefather et al., 1985 and Lancaster and Nicoll, 1987).

Ca-KCa VERSUS Na-KV ELECTROGENESIS

From the peak KCa current we evaluated that 25-30% of the available KCa conductance was activated during the spike, which is comparable with the data on V-dependent K channels in the squid axon (Bezanilla et al., 1970). Similarly, the Ca ion inflow per unit surface during one spike corresponds to a net transfer of charge of $3.7\ 10^{-12}$ positive charges/cm^2/spike, a figure closely related to the Na influx in sepia and squid axons: 3.5 to 4.5 10^{-12}mole/cm^2/spike.

These data show that a purely Ca-dependent spike such as that which exists in U cells is generated in a very similar way to that of the purely Na-dependent axon spike, in spite of intrinsic differences between the regulatory mechanisms operating in the two systems. It should be noted, however, that there is one order of magnitude in the time scale between the two spikes. Ca currents have slower activation kinetics than Na currents and there is a finite delay in the expression of KCa currents even when they work in the fast facilitated mode.

Ca DEPENDENT SIGNALS ARE FREQUENCY LIMITED

The dynamic properties of the Ca- and V-dependent controls of KCa channel opening provide clues to account for the spike attenuation and fast adaptation displayed by U cells. As reported in the preceding section, the first spike within a burst triggered a large repolarizing KCa current. In addition, it induced Ca accumulation in the peripheral cytoplasm. This elevated Ca maintained most KCa channels in the Ca-bound closed state ready to be opened by cell

depolarization. Therefore in the case of spikes occurring in this period, some of the KCa channels will have a pure voltage dependency and will open early in the depolarizing phase (Gola, 1987). This early opening will limit the expression of the Ca current and reduce the spike overshoot. The process will continue and result in spike blockade when early KCa currents surpass Ca currents. Since the decay of the Ca-bound state lasts about 1s, the attenuating process will be detectable at firing frequencies as small as 1-2 spike/s. Cells with purely Ca-dependent spikes have a low-frequency firing capability.

Ca-INDUCED BLOCKADE OF Ca-ACTIVATED K CURRENTS

The dual effects of Ca on outward currents have been well documented (Alkon and Sakakibara, 1985; Pallota, 1985; Tokimasa, 1985). In particular, in Helix neurons it has been shown that brief Ca entries produce a persistent block of part of the outward current (Heyer and Lux, 1976). The current suppressed by elevated $[Ca]_i$ has the properties of the Ca-dependent component . The blockade is particularly prominent in the Helix nerve cells identified as P cells and it accounts for the production of long-lasting depolarized plateaus (Gola, 1985). As shown in the preceding sections, this Ca-induced inactivation does not operate in U cells. Therefore two distinct inactivating and non-inactivating KCa currents are present in Helix neurons (see Fig. 2). If we leave this point aside, both channels share a number of identical properties:

- their V- and Ca-dependency are identical
- the inactivating KCa current may have two activating modes: slow and facilitated activations
- its elementary conductance is also in the range of 30pS
- both channels are blocked by extracellular TEA ($K_D \simeq 1mM$) and are insensitive to 4AP, apamin and intracellular TEA.

We thus conclude that these channels belong to the same class of small-conductance KCa channels. Therefore, all the data concerning the non-inactivating channels also apply to the inactivating channels. In particular, due to their voltage sensitivity, they are mainly involved in spike repolarization and they play no role in the long-lasting events following spike trains.

The inactivating KCa channels (i-KCa) always coexist with V-gated K channels. The functional meaning of this observation is obvious since we will show that the i-KCa current alone is unable to support the spike repolarization during a spike burst.

In order to study the properties of the i-KCa current we first blocked the KV component using intracellular TEA (intracellular injection of Cs^+ was also used to specifically block KV currents).

We routinely used the stimulation paradigm introduced by Brehm and Eckert (1978) in order to select cells with inactivating Ca-dependent KCa currents. A test pulse producing a clearly detectable KCa current (identified by its kinetic parameters and voltage dependency) was preceded by a short conditioning pulse aimed at producing a brief Ca entry. The spike interval was set at 0.8-1s, the period necessary for the fading of the facilitating effect exerted by elevated $[Ca]_i$ on KCa currents. In cells with i-KCa channels, the conditioning pulse produced a persistent blockade of the channels. The blockade was linearly related to the Ca influx induced by the conditioning pulse, i.e. it was typically U-shaped with maximum blockade occurring with +30 to +50mV prepulse.

The Ca-induced blockade has two main characteristics:

1- it is a slow process; development and recovery have time constants ranging from 0.6 to 4s (the inactivation time constant is directly related to the Ca inflow).

2- It has a high Ca-sensitivity i.e. blockade is induced by minute Ca entries.

In response to a brief Ca entry the i-KCa current undergoes a series of events: an immediate acceleration (fast facilitated mode) which vanishes in 0.5-1s as in the non-inactivating KCa current, a prominent depression and a slow recovery to the control level. Full recovery from inactivation requires tens of seconds and it parallels the slow $[Ca]_i$ decline of the bulk cytoplasm as detected with the Ca-sensitive dye Arsenazo III (see Fig. 7).

The comparative sensitivity of the Ca-dependent activating and inactivating processes was assessed by inducing small, long-lasting Ca entries (with 5-10s cell depolarization) either in P cells or in U cells. We noted that slight depolarizations from the -50mV holding

potential were effective in blocking the i-KCa current in P cells (Fig. 6). Half-blockade was produced by 10-15mV depolarization. Evaluation of the Ca current flowing during the depolarization showed that 1-3% of the total available Ca current of the cell blocked 50% of the i-KCa current. Under the same conditions, the non-inactivating KCa in U cells was not affected. Larger depolarizations, i.e. larger Ca entries were needed to induce the fast activated mode (Fig. 6). From both sets of data we have evaluated that the affinity of Ca for the inactivating binding site is one order of magnitude larger than the affinity of Ca for the activating site(s). However, the Ca binding reaction to the inactivating site is a slow process even in the presence of large intracellular Ca accumulation.

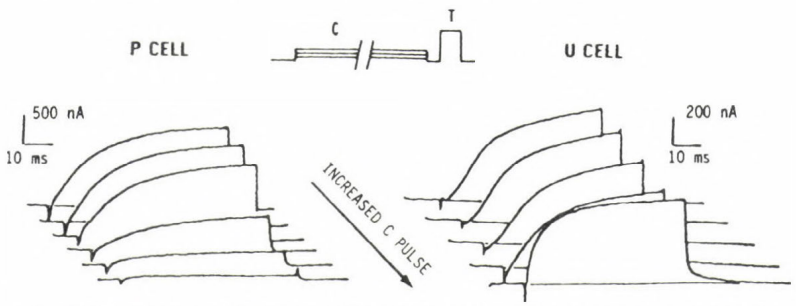

Fig. 6. Dual effect of Ca entries onto KCa channels. Intracellular Ca accumulation during long conditioning pulses (C) blocks KCa currents in P cell or speeds up its activation rate in U cell.

Therefore, in response to a brief, large Ca entry the i-KCa current will be transiently activated and further inactivated. Conversely, upon a slight and prolonged $[Ca]_i$ increase, the i-KCa channels will shift to the inactivated state without displaying any transient openings.

The high Ca sensitivity of the inactivating process was confirmed by the direct measurement of $[Ca]_i$ changes using the Ca-sensitive dye Arsenazo III. The dye was intracellularly injected, and its Ca-bound ratio was optically monitored. Arsenazo signals (indicating intracellular Ca accumulation) in response to step depolarizations are shown in Fig. 7. The signals were detectable with very slight (10-12mV) depolarizations which were effective in blocking i-KCa

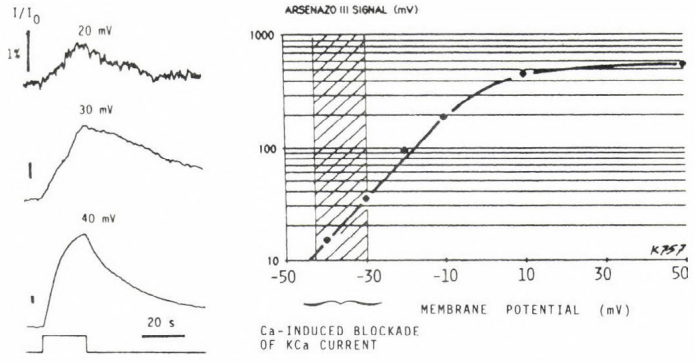

Fig. 7. Intracellular $[Ca]_i$ changes monitored with Arsenazo III. Diagram: plot of the Arsenazo signal versus membrane potential. The hatched surface corresponds to the membrane potentials which induce Ca-dependent i-KCa inactivation.

channels. From the large signals obtained at positive potentials we confirmed that half-blockade of the i-KCa current required minute Ca entries representing 1-3% of the Ca current capability of the cell.

CYCLIC AMP-INDUCED i-KCa BLOCKADE

It has been reported that intracellular injection of cAMP may induce a rise in intracellular free calcium (Connor and Hockberger, 1984). We made use of this property to further analyse the Ca regulation of KCa channels. By coupling the Arsenazo detection method with the intracellular injection of the nucleotides, we first confirmed that cAMP specifically increases the free Ca content in Helix neurons. Recordings from such an experiment are shown in Fig.8a where two successive Arsenazo signals occur in response to cell depolarization and cAMP injection, respectively.

The cAMP-induced $[Ca]_i$ increase was very specific. It was not induced by AMP, ATP, GTP and dibutyryl-cAMP. CPT-cAMP (a phosphodiesterase resistant derivative) and cGMP were less potent than cAMP. The phosphodiesterase inhibitor IBMX prolongs the cAMP-induced signal whereas the phosphodiesterase activator imidazole had the opposite effect. During these experiments we could not assess the changes in the KCa current since this current was partly blocked by Arsenazo. This blockade probably results from an EGTA-type effect

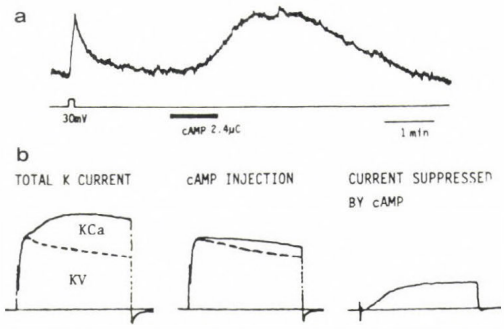

Fig. 8. cAMP induced blockade of i-KCa current. a) $[Ca]_i$ increase induced by cell depolarization and electrophoretic cAMP injection. b) cAMP injection in P cells, specifically reduces the slow KCa component of the compound KCa and KV outward currents.

due to the Ca-chelating properties of the dye. The above experiments were therefore performed before and after the dye injection. The results showed that cAMP did not affect the non-inactivating KCa current in U cells (large injections of the nucleotide had a blocking effect which actually resulted from that of the Ca current). By contrast, cAMP blocked part of the outward current in P cells. The blocked part corresponded to the i-KCa current component and the non-blockable part to the V-gated component (Fig. 8b). The blockade had the same sensitivity to various nucleotides and inhibitors as the Arsenazo signal. The time courses of blockade and recovery after the cAMP injection paralleled the change in $[Ca]_i$ monitored by Arsenazo. These results provide strong confirmation of our previous conclusion that i-KCa channels are tightly controlled by slight $[Ca]_i$ changes via a high affinity binding site linked to the inactivating process.

CONCLUSION

From the above data we were able to obtain a relative estimate of the Ca sensitivity of the 3 main processes resulting from a rise in cytosolic free calcium:

inactivation of i-KCa channels > inactivation of Ca channels > activation of i-KCa channels

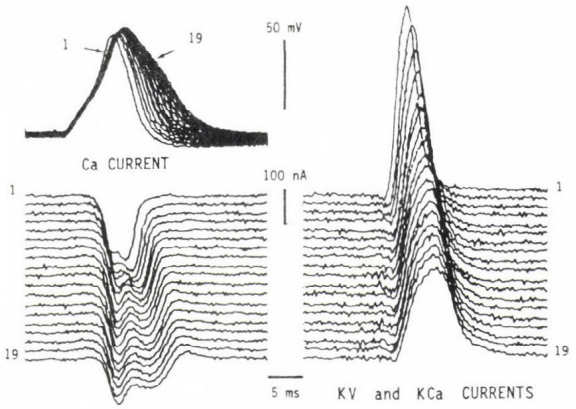

Fig. 9. Changes in Ca and K currents during a P cell spike burst (bursts of 19 spikes fired at 7Hz).

This hierarchy seems to imply that in cells governed by Ca and i-KCa channels only, the stabilizing feedback controls exerted by the entry of calcium can be surpassed by the destabilizing effect of the Ca-sensitive, long-lasting KCa channel blockade. Except in P cells, this is not so, however, since i-KCa channels always coexist with KV channels which provide the cell with an additional repolarizing mechanism. In P cells, the KV channels have a very pronounced voltage-controlled inactivating system with kinetic parameters closely resembling those of the i-KCa channels. Therefore, during sustained stimulation both K channels will be progressively depressed, enhancing the depolarizing tendency of sustained Ca currents and finally leading to paroxysmal-type depolarizations. These events are illustrated in Fig.9 which gives the underlying Ca and K currents that have been determined during the spike lengthening preceding the transition to the plateau phase. This figure exemplifies the dramatic decrease in the repolarizing currents when the various processes depicted in Fig. 1 act synergically with conventional voltage-dependent inactivation.

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PRE- AND POSTSYNAPTIC EFFECTS OF CAPSAICIN ON SNAIL NEURONS

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INTRODUCTION

The action of capsaicin (CAP) on synaptic transmission and transmitter--evoked events has not been well elucidated. However, it is generally accepted that the drug can deplete substance P (SP) and some other peptides from unmyelinated primary sensory neurons in mammals (3, 10, 14, 28). Tsunoo et al. (29) found that the non-cholinergic slow EPSP was totally blocked by a low dose of CAP in guinea pig sympathetic ganglia. Similar observations were made by Dun and Király (3), Urbán et al. (30) and Randić and Urbán (20). Earlier, we reported that CAP attenuated a synaptically activated potassium conductance and an ACh-evoked depolarization in snail neurons (5, 8).

Some observations suggest a possible interaction of CAP with putative transmitters. Göres and Jung (12) stated that CAP inhibited the pressor reflex evoked by ACh from the perfused small intestine. Salt and Hill (25) found that CAP potentiated the excitatory responses evoked by glutamate and aspartate in the trigeminal nucleus caudalis. Williams and Zieglgänsberger (32) noted a suppression of GABA-evoked depolarization by CAP in the dorsal root ganglion cells, but the glutamate-evoked excitatory effect was not significantly affected by the drug. Hajós et al. (13) reported an impairing effect of CAP with serotonin on thermosensitive structures in the preoptic region of the hypothalamus of rats.

The aim of our study was to characterize the pre- and postsynaptic effects of CAP through the use of stimulus-evoked excitatory and inhibitory postsynaptic currents (EPSCs or IPSCs) and transmitter-induced depolarizing and hyperpolarizing responses.

MATERIALS AND METHODS

The experiments were performed on identified and unidentified neurons of the snail Helix pomatia L. Among the identified neurons, the metacerebral giant cell (MCC) and the right parietal burster (RPal) were used (2, 21). The preparation, the physiological solution and the recording procedure were the same as described earlier (7, 8). Bipolar teflon-coated silver wires were used for stimulation of the major nerves (anal, intestinal, left and right parietal or connectives) of the ganglia. The resulting potential or current signals were recorded on a Tektronix storage oscilloscope and photographed from the screen, or a chart writer (Phillips PM 8202) was alternatively used. The stimulus-evoked or transmitter-induced current amplitudes were measured in at least three different membrane potentials in each experiment. Conductance was obtained as the slope of the current-voltage (I-V) relations, determined by linear regression. The reversal potential was obtained by linear interpolation between measured inward and outward current values. Transmitters were bath-applied by use of the fast perfusion system. CAP was also bath-applied in the perfusion solutions. Sometimes, high Mg (20 mM) and high Ca (20 mM) or high Mg (40 mM) and low Ca (5 mM) solutions were used to isolate monosynaptic connections during nerve stimulation or to decrease the indirect influence when transmitters were applied. Low potassium (1 mM) solution was also used to linearize the I-V relations. Mean and standard deviations of the values were calculated and the test responses with the controls were compared by use of Student's t test. All experiments were performed at room temperature (22-25 °C) with active snails.

RESULTS

The effects of CAP on excitatory postsynaptic currents

On stimulation of the nerves of the suboesophageal ganglia, various types of EPSCs can be recorded in different neurons bathed in normal, in high Mg and high Ca or in high Mg and low Ca solutions. The study showed that the stimulus-evoked transmitter release was not influenced by CAP in most of the synapses. However, CAP decreased or blocked the stimulus-evoked EPSCs in some other synapses. The result of a typical experiment is shown in Fig. 1, where 200 μM CAP reversibly blocked a fast EPSC (Fig. 1 A). The ampli-

tude of the stimulus-evoked EPSCs sometimes increased (Fig. 1 B). The amplitude and occurrence of spontaneous EPSCs were generally not influenced by CAP, but they were sometimes suppressed, as shown in Fig. 4 A. Rarely, CAP facilitated the spontaneous EPSCs in certain synapses.

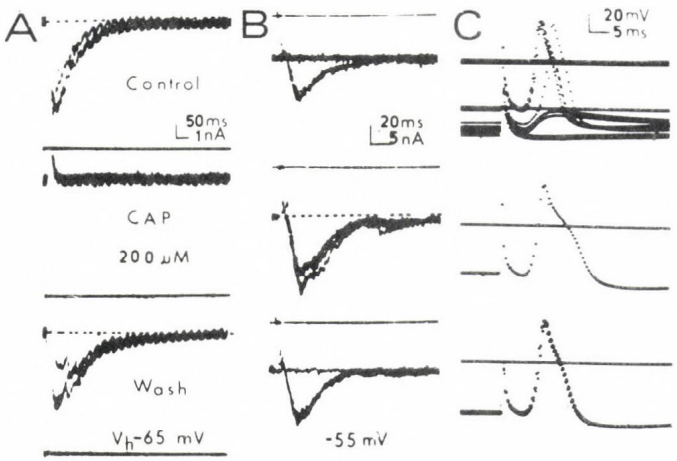

Fig. 1. CAP-induced suppression (A) and facilitation (B) of stimulus--evoked EPSCs, and its action on antidromic spikes (C).

Evidence has been presented that CAP can induce an axonal conduction block in mammalian nerves and crayfish giant axons (26, 33). As shown in Fig. 1 C, CAP (200 µM) did not influence the spike latency of an antidromic action potential, but it reversibly prolonged the action potential duration. Other observations on various antidromic spikes gave similar results. The depression of the EPSCs may therefore not be due to a blockage of axonal conduction. The antagonism between CAP and excitatory transmission is most probably due either to a block of the presynaptic release mechanism or to a suppression of the postsynaptic excitation. The former possibility is less likely, but it is not totally excluded, because CAP attenuates the Ca--current in a normal Ca-containing solution in some snail neurons (7), whereas this effect of the drug is only moderate in a high Ca medium (8). Accordingly, the depression of excitatory transmission by CAP is most probably due to its postsynaptic effect.

The postsynaptic effects of CAP on excitatory transmitters

Two identified neurons (MCC and RPal) which respond to a variety of trans-

mitters with depolarization and an increase of the membrane conductance were used in much of the work (17, 23). Low potassium saline was used in the experiments to linearize the I-V relations over the -30 to -100 mV membrane potential range, which permitted better quantitative analysis.

Dopamine (10^{-5} M) and noradrenaline (10^{-5} M) - induced depolarizations or inward currents were not influenced by even a high dose of CAP (300 µM) in the MCC. However, CAP depressed the depolarization and associated conductance increase to GABA, 5-HT and ACh in both identified neurons. Figure 2 A shows the time course of CAP action on the relative amplitude of the GABA--induced inward current. GABA evoked excitation due to an increase of Cl^- conductance. CAP caused a depressant action on the ACh-induced and Cl^---mediated excitation in a neuron of the visceral ganglion as well. [For a review of the transmitter actions on snail neurons, see (11).] As can be observed in Fig. 2 B, extrapolated reversal potentials of the ACh and 5-HT inward currents show a similar ion mechanism, which corresponds with a conductance increase for both Na^+ and K^+ in the MCC. Equimolar 5-HT caused a smaller conductance increase than ACh did. Nevertheless, CAP attenuated both the ACh and the 5-HT-induced inward current in a dose-dependent, reversible manner. CAP decreased the conductance change during the actions of ACh and 5-HT, and increased the time to peak of the ACh-evoked inward current with a possible interaction of the activation kinetics. The dose-inhi-

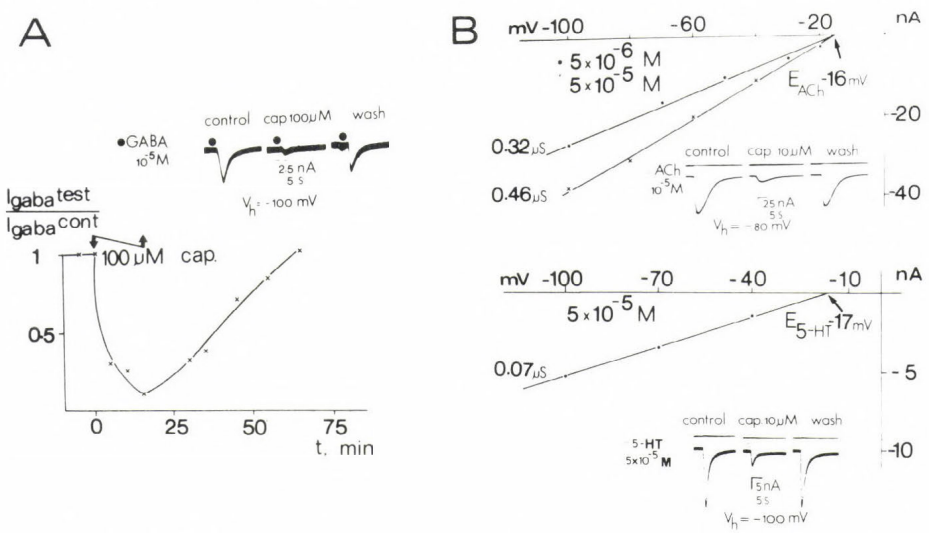

Fig. 2. CAP reversibly attenuates GABA (A), ACh and 5-HT-evoked inward currents (B) in the MCC.

bition relationship of CAP on the inward ACh current is shown in Fig. 3 A, while the double-reciprocal plot of the values is presented in Fig. 3 B. The Lineweaver-Burk plots of the ACh, GABA and 5-HT dose-inhibition data were used to estimate the dissociation constants (K_d) for CAP in the MCC. K_d values for the effects of CAP on the 5-HT, ACh and GABA responses were 1, 5 and 6.7 μM, respectively.

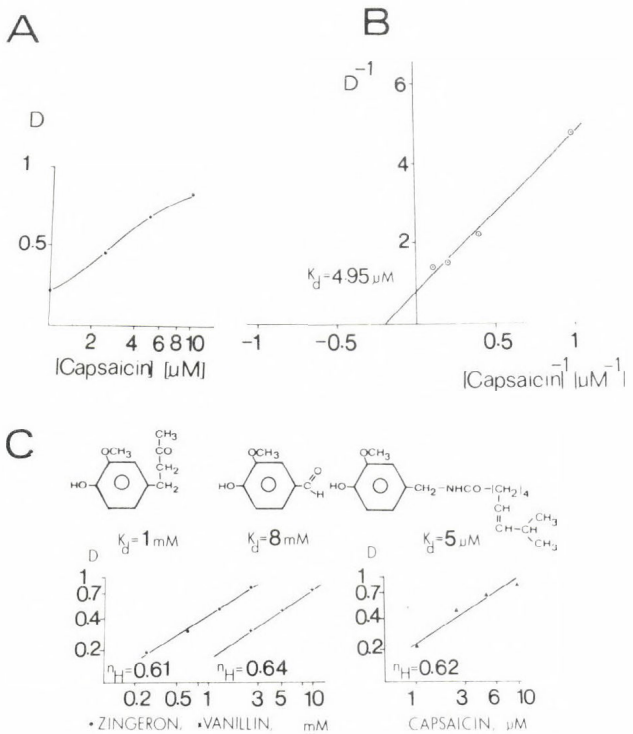

Fig. 3 A. Dose-inhibition curve for the action of CAP on the ACh-evoked inward current ($D = 1-I_{ACh\ test}/I_{ACh\ control}$). B. Lineweaver-Burk plot of the effect of CAP on ACh inward currents. The reciprocal of D was plotted against the reciprocal of the CAP concentration. C. Hill plots of the dose--inhibition data for zingeron, vanillin and CAP on ACh inward currents.

Structure-action analysis of the effects of CAP and related compounds on ACh-evoked inward current

The analysis of CAP and related compounds such as vanillin and zingeron on ACh-evoked currents showed that the K_d values of the compounds increased

with decreasing alkyl tail length of the molecule. The K_d values for vanillin and zingeron were 8 and 1 mM, respectively. The dose-inhibition data for vanillin, zingeron and CAP are shown on a double-logarithmic scale in Fig. 3 C. The Hill plots were used to estimate the Hill constants (n_H) of each compound, and these proved to be similar for the three molecules in the study. The analysis shows a one-to-one binding stoichiometry for vanillin, zingeron and CAP.

The effects of CAP on inhibitory postsynaptic currents

It is known that the RPal burster receives inhibitory inputs from the periphery (9, 19, 22, 24). On stimulation of the anal, intestinal, left and right parietal nerves and connectives, monosynaptic IPSPs were reported, whose ion-dependency and pharmacology were also studied (18, 31). Electron-microscopic observations showed that this cell receives peptidergic synaptic contacts (4).

CAP differentially influenced the IPSCs recorded on nerve stimulation. The IPSC recorded on stimulation of the intestinal nerve was not influenced by the drug, whereas the response showed a K-dependence and the potassium ion conductance increase was responsible for the stimulus-evoked inhibition, while CAP markedly decreased the voltage-gated potassium currents of the neuron (6). The amplitude of the IPSC recorded at -40 mV holding potential was 18.8 ± 1.7 nA (mean $\pm$ S.D., n = 6) under control circumstances, and 17.9 ± 0.7 nA (n = 9) in the 200 /uM CAP-containing solution.

The IPSC recorded on stimulation of the left cerebro-pleural connective (l.c.-pl.c.) proved to be CAP-sensitive and decreased by about 50 % in 300 /uM CAP-containing solution (Fig. 4 A). A similar decrease of the spontaneous EIPSCs gradually developed during CAP treatment. The amplitudes of the stimulus-evoked and spontaneous EIPSCs depended markedly on the membrane potential, but a sharp reversal potential on the voltage axis of the I-V curve was not reached. Experiments with potassium channel blockers showed differential modulation of the synaptic transmission in this synapse. Ba^{++} (7 mM) increased the amplitude of the stimulus-evoked IPSCs by about 40 % and markedly enhanced the amplitude and occurrence of the spontaneous EIPSCs. Thus, Ba^{++} behaved as a secreteger. TEA^{+} and 4-AP decreased the stimulus-evoked outward currents by 20 % and 75 % in 12 and 5 mM concentrations, respectively (Fig. 4 B).

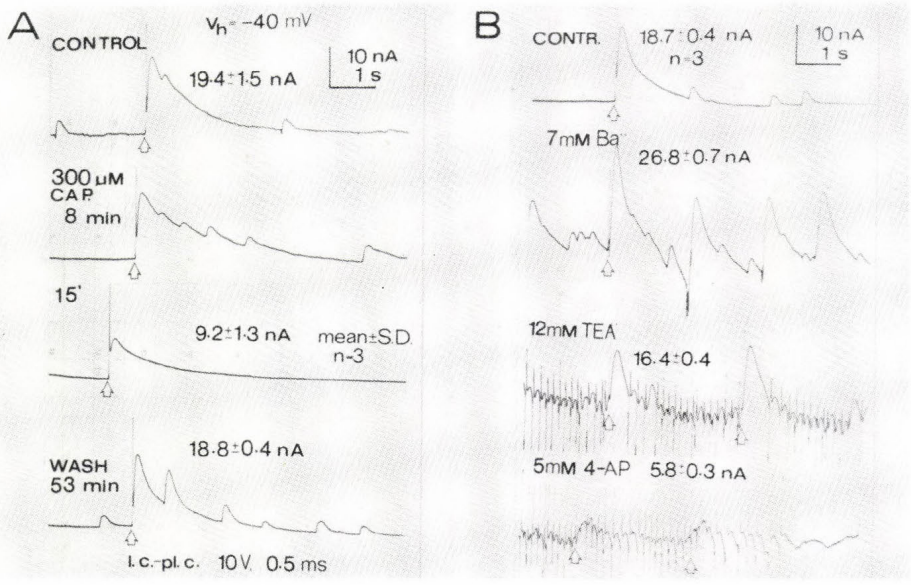

Fig. 4. Effects of CAP (A) and potassium channel blockers (B) on IPSCs recorded after stimulation (arrowheads) of the left cerebro-pleural connective (l.c.-pl.c.).

The IPSCs recorded on stimulation of the left parietal and anal nerves increased by about 50 % and 87 % respectively, in 300 µM CAP solution. The amplitude of the evoked IPSP after stimulation of the left parietal nerve was 15.1 $\pm$ 2.1 mV (n = 6) in the control solution. CAP (300 µM) significantly increased the amplitude of the response to 21.2 $\pm$ 1.3 mV (n = 7), the value returning to 13.1 $\pm$ 1.3 mV (n = 7) after a 40-min washing. As Fig. 5 shows, CAP increased the amplitudes of both the fast and the slow IPSCs activated by stimulation of the anal nerve at each holding potential, without change of the reversal potentials in normal solution. The slope conductances of the fast and the slow components of the IPSCs are 0.41 and 0.25 µS, respectively, under control circumstances. CAP (300 µM) increased the slope conductance of the $IPSC_f$ to 0.83 and that of the IPSCs to 0.49 µS, the initial levels being recovered slowly during a long washing period (more than 1 h). The amplitude and number of similar two-phasic spontaneous IPSCs were sometimes reversibly increased in CAP-containing solution as well. The experiments performed with potassium channel blockers

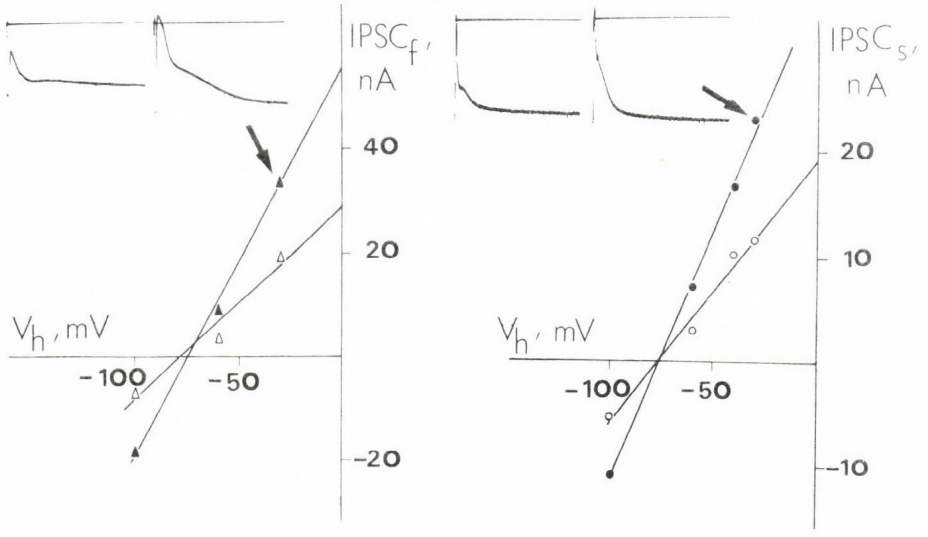

Fig. 5. CAP (300 /uM) facilitates both the fast and the slow components of the IPSCs recorded on stimulation of the anal nerve at each holding potential.

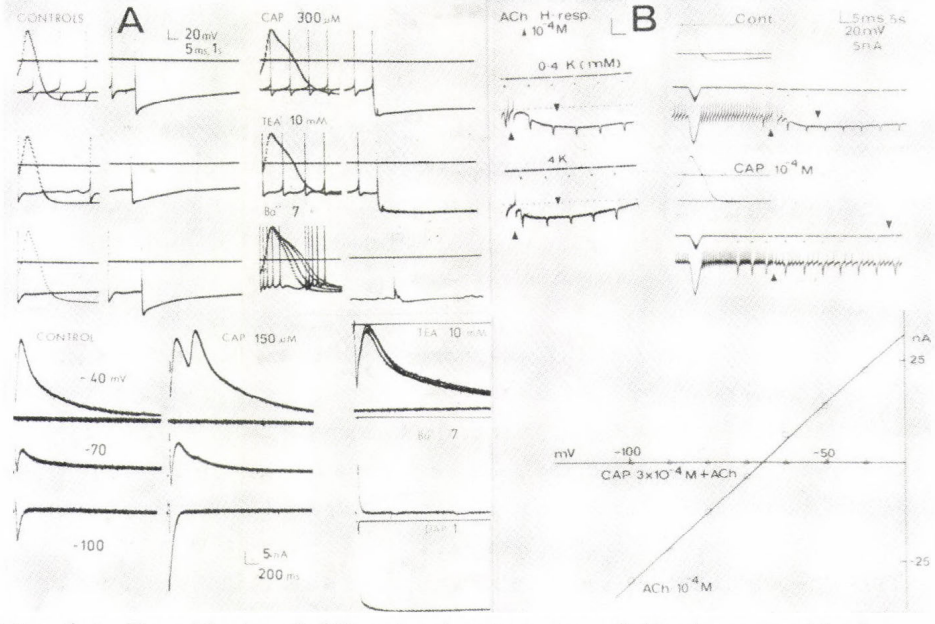

Fig. 6 A. The effects of CAP and potassium channel blockers on stimulus--evoked IPSPs and IPSCs in the RPal burster after stimulation of the anal nerve. B. CAP blocks the potassium-dependent H-response and the corresponding conductance increase in unidentified neurons.

showed that TEA^+ (10 mM) increased the amplitude and duration of the same or similar type of IPSCs in the burster or in one of the adjacent neurons. However, Ba^{++} (7 mM) and 3,5-diaminopyridine (1 mM) blocked them (Fig. 6 A). The IPSCs recorded in the MCC after stimulation of the labial nerve were not influenced by CAP, but an ILD evoked by stimulation of the l.c.-pl.c. decreased by about 80 % in 200 µM CAP solution in a neuron of the upper part of the right parietal ganglion.

Effects of CAP on inhibitory transmitters

CAP proved to be surprisingly ineffective on most of the inhibitory transmitters studied. Thus, the FMRFamide and L-glutamate-evoked outward currents and the corresponding increase of the potassium conductance (g_k) were not influenced by the drug in the MCC. The amplitudes of the 10^{-4} M glutamate and 10^{-5} M FMRFamide-induced outward currents at -45 mV holding potential were 4.9 ± 0.4 nA (n = 3) and 8.7 ± 1.1 nA (n = 3) in low potassium solution. CAP (150 µM) decreased the amplitude of the glutamate-evoked outward current to 4.6 ± 0.3 nA (n = 3), while 300 µM CAP increased that of the FMRFamide-induced response to 9.3 ± 1.6 nA (n = 3), nonsignificantly. The 5-HT and L-glutamate-evoked hyperpolarizations were not influenced by CAP in an unidentified cell adjacent to the MCC. CAP had no influence on the Cl^--dependent hyperpolarization evoked by ACh, but the potassium-dependent hyperpolarization was markedly suppressed in some unidentified neurons. This is shown in typical experiments carried out under current clamp and voltage clamp circumstances (Fig. 6 B).

DISCUSSION

The study of various stimulus-evoked EPSCs and IPSCs showed that the spontaneous and evoked release of transmitters were selectively modulated by CAP in the snail brain neurons, because the drug had facilitatory and depressant actions on a limited number of excitatory and inhibitory synapses.

The facilitation of some stimulus-evoked responses may be a presynaptic and TEA^+-like action of the drug. Within the mammalian neuropharmacology of CAP, the liberation of SP and some other peptides from unmyelinated primary sensory neurons is well documented (3, 10, 16) and may indicate the involvement of peptide transmitters in the facilitation process in

this preparation as well. The postsynaptic effects of most of the inhibitory and some of the excitatory transmitters studied were not influenced by CAP treatment. However, an enhanced liberation of any one of them during presynaptic facilitatory action of the drug might increase the stimulus--evoked response. This is the case with SP in mammals, where CAP does not influence the peptide induced depolarization (3, 20, 29). Electron-microscopic observations indicate that certain peptides may be involved in the inhibitory modulation of the RPal burster activity (4), and it cannot be excluded that CAP may liberate some peptide from them. Identification of the actual transmitter(s) needs further efforts.

The depressant action of CAP found in some excitatory and inhibitory synapses is most probably a postsynaptic action of the drug. The transmitter application study suggested that CAP attenuated the 5-HT, ACh and GABA--evoked excitatory responses. Thus, CAP can block cholinergic, gabaergic and serotoninergic EPSCs, in accordance with the postsynaptic mechanism of its action. It appears that the CAP-sensitivities of the ligand- and voltage-gated channels differ markedly, because the 5-HT, ACh and one GABA--evoked responses were suppressed in /uM doses, while one hundred times higher concentrations of the drugs were necessary to attenuate the membrane ionic currents in the MCC (8). It seems that the action of CAP on the ACh responses is a molluscan-specific event, for the fast cholinergic EPSP was not influenced by the drug in the guinea pig sympathetic ganglia, similarly to the homologous Cl^- -dependent hyperpolarization in the snail (3, 29). Furthermore, it was found that CAP had no effect on the cholinergic contraction or adrenergic relaxation induced by transmural electrical stimulation in the rabbit ileum (1). Our results with 5-HT and GABA suggest a general interaction of CAP with these transmitters, as a similar suppression of 5-HT and GABA responses has already been reported in rats (13, 32). CAP was found to have an aminopyridine-like action in the synapses, where the IPSCs were attenuated. Apart from the potassium-dependent ACh H--response, it is not clear which types of transmitter actions are suppressed by CAP, because ACh is not a candidate of any of the responses recorded in the RPal burster after nerve stimulation. The experiments with CAP and other potassium channel blockers showed that the ligand-gated potassium channels differ from the voltage-gated ones in pharmacological properties.

Analysis of the structure-action relationship of CAP and related compounds (vanillin, zingeron) on the ACh D-response in the MCC showed that the va-

nillyl head of the molecule may be responsible for binding to the recognition site with a one-to-one stoichiometry (n_H = 0.6) for all three compounds. However, the K_d values of the molecules increased with shortening of the alkyl tail of the compounds (K_d = 5 μM, 1 and 8 mM for CAP, zingeron and vanillin). Thus, the blocking ability of the CAP molecule as an ACh antagonist increased considerably in parallel with the acylamide-alkyl chain length of the drug. The results of the structure-action experiments are in agreement with previous observations made with various CAP congeners, which showed that modification of the CAP molecule markedly modulated the effectiveness of the compound (15, 27).

SUMMARY

It was shown that CAP differentially modulated the stimulus-evoked EPSCs and IPSCs or transmitter-evoked depolarizations and hyperpolarizations. A presynaptic TEA^+-like action of the drug can facilitate the transmission in both certain excitatory and inhibitory synapses. The postsynaptic attenuation of the 5-HT, GABA and ACh-evoked excitatory responses and the aminopyridine-like suppression of the ligand-gated potassium current may be responsible for the depressant action of CAP in some synapses. Structure-action analysis with CAP, vanillin and zingeron on the ACh-evoked inward current showed the decreasing effectiveness of the drugs with shortening of the alkyl tail of the molecules.

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DISCUSSION

ARVANOV, V.: How can you explain your data that capsaicin in some EPSCs induced inhibitory and in others EPSC-facilitatory effect?

ERDÉLYI, L.: I think that the two types of effects of CAP on EPSCs highly relate to the nature of transmitters involved.

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CONCENTRATION CLAMP ANALYSIS OF THE GABA-INDUCED CHLORIDE CURRENT IN ISOLATED APLYSIA NEURONS

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SUMMARY

1. The chloride current (I_{Cl}) induced by gamma-aminobutyric acid (GABA) in the isolated neurons of circumesophageal and abdominal ganglia of Aplysia kurodai was studied using the 'concentration-clamp' technique, which combines the internal perfusion and the rapid exchange of the external solution within a few msec.

2. The GABA-I_{Cl} was rapidly activated and then decayed (desensitized) during a maintained presence of the agonist.

3. The dose-response curve for the GABA-I_{Cl} was sigmoidal with a K_d of 6.4×10^{-5}M and a Hill coefficient of 0.88.

4. The peak conductance elicited by GABA was voltage-independent. The activation and desensitization kinetics were also insensitive to voltage.

5. Recovery from desensitization was double exponential and it was almost complete within two minutes.

6. The GABA-I_{Cl} cross-desensitized with that evoked by muscimol or β-alanine.

7. Pentobarbital inhibited the GABA-I_{Cl} non-competitively in a dose-dependent manner. Diazepam had no effect on the GABA-I_{Cl}.

INTRODUCTION

Neurons of the mollusc Aplysia have membrane receptors and associated ionic channels that are gated by a variety of neurotransmitter substances (Kehoe, 1972; Gerschenfeld & Paupardin-Tritsch, 1974; Swann & Carpenter, 1975; Yarowsky & Carpenter, 1976, 1978; Sawada et al., 1984; Gaillard & Carpenter, 1986). Individual neurons not only have receptors for multiple

neurotransmitters, but may also have multiple receptors with different associated conductance mechanisms for a single transmitter (Gardner & Kandel, 1972; Kehoe, 1972; Gaillard & Carpenter, 1986). Kinetic studies with perturbation or fluctuation analysis are indispensable to elucidate the mode of interactions between the transmitter and the receptor channel complex. With a conventional drug application method such as bath perfusion, the peak amplitude of a current response is obscured by desensitization. With iontophoretic or pressure application, it is impossible to determine the concentration of an agonist at the receptor site. In the present work we have studied the kinetics and pharmacological properties of the GABA-induced Cl^- conductance under voltage-clamp using the 'concentration clamp' technique. The technique allowed accurate description of GABA dose-response characteristics, the voltage-dependence, and the kinetics of activation and desensitization.

METHODS

Preparation. All experiments were performed on voltage-clamped isolated neurons of circumesophageal and abdominal ganglia of the marine mollusc Aplysia kurodai. The neuronal somata were isolated by enzymatic and mechanical treatments as described previously (Ikemoto et al., 1987). Details of the intracellular perfusion procedures have been described previously (Akaike et al., 1985). Non-identified isolated neurons having a diameter of about 50 μm were used in the present experiments.

Solution. The standard bathing-perfusion medium, artificial sea water (ASW), contained (mM): NaCl 450, KCl 10, $CaCl_2$ 10, $MgCl_2$ 55 and Tris 10 (pH 7.8 with HEPES and Tris base). To isolate Cl^- current from Na^+ and K^+ currents, Na^+ and K^+ in both external and internal solutions were replaced with $Tris^+$ and Cs^+, respectively. The ionic composition was (in mM): external, Tris-Cl 340, Tris-base 100, CsCl 10, $CaCl_2$ 10 and $MgCl_2$ 55 (pH 7.8); internal, Tris-base 300, aspartic acid 270, CsCl 300 and EGTA 5 (pH 7.2). pH of the test solutions was adjusted with HEPES and Tris-base. All experiments were performed at room temperature (about 20 °C).

Rapid drug application using the 'concentration clamp' technique. The 'concentration clamp' technique was developed to enable an extremely rapid application or 'switching' of the external solution containing drugs (Akaike et al., 1986; Inoue et al., 1986; Akaike et al., 1987). The cell attached tip of the suction pipette was inserted into a plastic tube through a circular hole of about 500 μm in diameter (Akaike et al., 1987).

The lower end of this tube was directly exposed to the external test solution by moving the stage vertically, on which the solution-containing dishes were placed. A negative pressure of 3 cm Hg applied to the upper end of the plastic tube was controlled with an electromagnetic valve, driven by 24 volt D.C. supplied by a stimulator (Nihon Kohden, SEC-7103). The time constants of the solution exchange was about 2 msec for the isolated Aplysia neurons.

Electrical measurement. The membrane potential was controlled by a single-electrode voltage-clamp system (Ishizuka et al., 1984). The resistance of the suction electrode filled with standard internal solution was about 400 KΩ. The current and voltage were monitored on a storage oscilloscope (Tektronix, 5113) and on an ink recorder, and were also digitized and stored on magnetic tapes for later analysis. Curve fittings were performed using microcomputer (PC-98XA, NEC, Japan).

Drug. Drugs employed in the present experiments were: dispase (10,000 protease unit/5 ml, Godo Shusei, Japan), GABA (Tokyo Kasei), β-alanine (Ishizu), muscimol (Sigma), pentobarbital (Tokyo Kasei), α-chloralose (Wako Pure Chemical Industries, Ltd.) and bicuculline (Sigma). Diazepam was kindly presented by Yoshitomi Pharmaceutical Company.

RESULTS

Recovery from desensitization

Application of GABA to isolated Aplysia neurons elicited a I_{Cl} which declined completely during a maintained presence of the agonist. In order to avoid accumulation of the receptor-channel complex in a desensitized state in experiments requiring repeated agonist application, it was necessary to first examine the time course of recovery from desensitization. Paired applications of GABA with varying wash intervals showed that recovery was double exponential and nearly complete within two minutes. On this basis GABA was applied with a 3 minute interval unless otherwise noted.

Concentration-dependence of GABA-induced current

The GABA-I_{Cl} increased steeply in amplitude with increasing the concentration from 6 x 10^{-7}M up to 10^{-3}M, which gave a nearly maximal response. Further increase to 10^{-2}M gave little additional increase in the amplitude. The relationship between the peak amplitude and the GABA concentration is shown in Figure 1, in which all responses are normalized to the peak current produced by 10^{-4}M GABA. The dose-response

relationship accorded with the conventional expression:

$$I = I_{max} \cdot \frac{C^n}{C^n + K_d^n} \quad \cdots\cdots\cdots \quad (1)$$

where I is the observed GABA-I_{Cl}, I_{max} the maximum current, C the GABA concentration, K_d the dissociation constant, and n the Hill coefficient. A least square fitting gave a Hill coefficient of 0.88 and a K_d of 6.4 x 10^{-5} M. The continuous line in Figure 1 was drawn according to Eqn. (1) using those values and I_{max}=1.7.

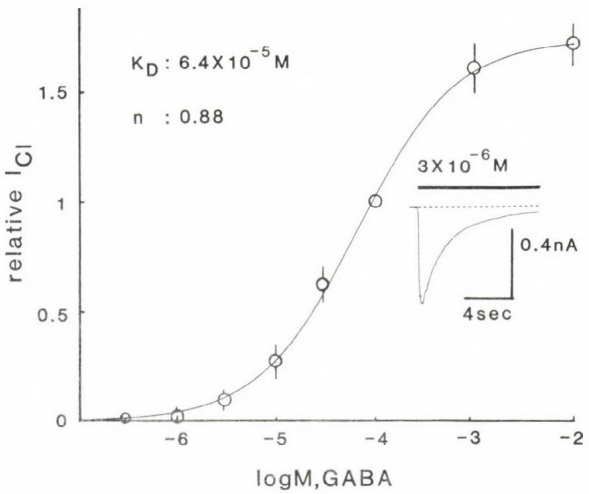

Fig. 1 Dose-response curve for GABA. The peak I_{Cl} was normalized to that evoked by 10^{-4}M GABA. External and internal Cl ions were 480 and 300 mM, respectively. The continuous theoretical curve was drawn according to Eqn. (1). Inset, a I_{Cl} induced by 3 x 10^{-6}M GABA in a neuron voltage-clamped at -20 mV. The neuron was exposed to GABA for the period indicated by a horizontal bar above the response. Each point is the average of 5 to 7 experiments. Vertical bars indicate one standard error of mean.

Voltage-dependence

The peak conductance elicited by GABA was voltage independent. This was shown by the linearity of the I-V relationship. The reversal potential for the GABA-I_{Cl} obtained from the intercept of the I-V curve on the voltage-axis was about -7.1 mV, which is close to the Cl^- equilibrium potential of -10.7 mV, calculated from the Nernst equation.

Concentration- and voltage-dependence of activation and desensitization

The activation phase was single exponential at all concentrations of GABA. The time constant decreased with increasing concentrations of GABA, but it was independent of the membrane potential (Fig. 2).

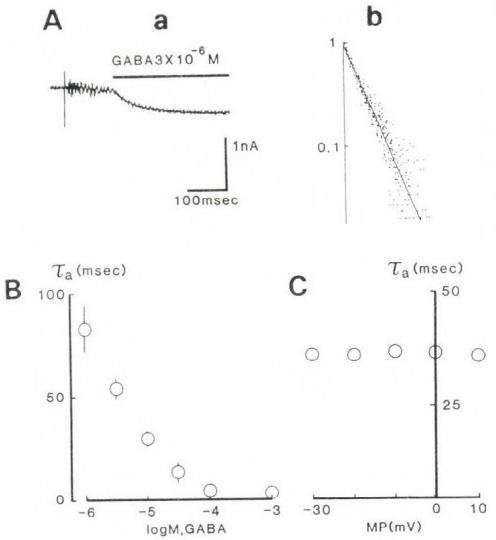

Fig. 2

A: A I_{Cl} induced by 3 x 10^{-6}M GABA (a), and the semilogarithmic plot of the rising phase of the current (b).

B: Dose-dependence of activation time constant (τ_a).

C: Voltage-dependence of τ_a.

Desensitization proceeded double exponentially but at low and high concentrations one component was obscured. The time constants became smaller depending on the concentration, whereas they were voltage-independent (Fig. 3). Recovery from desensitization was also double exponential with time constants of 8.5 and 50 seconds (not shown).

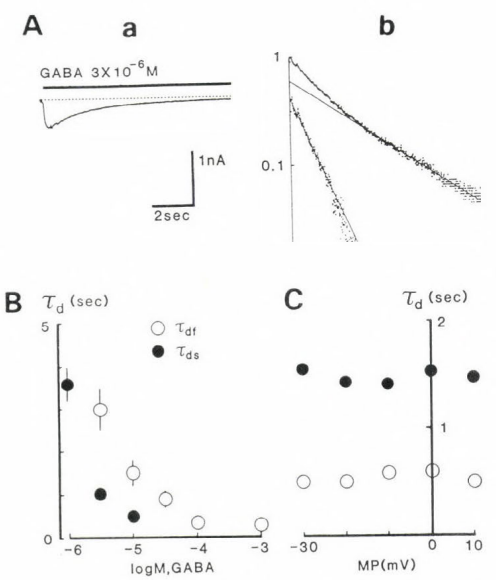

Fig. 3

A: A I_{Cl} induced by 3 x 10^{-6}M GABA (a) and the semilogarithmic plot of the desensitization phase (b).

B: Dose-dependence of the two time constants of desensitization (the fast and the slow, τ_{df} and τ_{ds}).

C: Voltage-dependence of τ_{df} and τ_{ds}.

GABA-related compounds

Muscimol and β-alanine also induced a I_{Cl} which desensitized completely. When the extensive desensitization was produced by either muscimol or β-alanine, subsequent application of GABA did not evoke any response, indicating a cross-desensitization (Fig. 4A).

The GABA-induced responses of frog sensory neurons was suppressed almost completely by 10^{-5}M bicuculline (Akaike et al., 1987; Yakushiji et al., 1987). The GABA response in Aplysia neurons was, however, little inhibited even by a high concentration of 10^{-4}M (Fig. 4B).

In the sensory neurons of the frog (Nicoll, 1975; Akaike et al., 1985) rat (Evans, 1979; Connors, 1981) and cat (Higashi & Nishi, 1982) and in the hippocampal pyramidal cells of the mouse (Alger & Nicoll, 1982), the GABA-I_{Cl} is enhanced by pentobarbital. Diazepam, one of the benzodiazepine derivatives, also enhances the GABA-I_{Cl} in cultured mammalian neurons (MacDonald & Barker, 1978; Choi et al., 1981; White et al., 1981; Chan et al., 1983), frog sympathetic ganglion cells (Suria & Costa, 1975), afferent fibers to the rat cuneate nucleus (Simmonds, 1980), and CNS neurons (Curtis et al., 1976; Raabe & Gumnit, 1977). In the present experiments, however, diazepam had no effect on the GABA-I_{Cl} while pentobarbital depressed the response dose-dependently in a non-competitive manner (Fig. 5).

DISCUSSION

Hill coefficients of near 2 for the GABA-activated Cl^- conductance have been reported in a variety of preparations from both vertebrates (Sakmann et al., 1983; Bormann & Claphan, 1985; Akaike et al., 1986; Kaneko & Tachibana, 1986; Randle & Renaud, 1987) and invertebrates (Smart & Constanti, 1986). An exception was a value of less than unity reported by Matsumoto et al. (1986) for the GABA-I_{Cl} in cells of the abdominal ganglion of Aplysia kurodai. Using the isolated neurons of Aplysia kurodai, we confirmed the results by Matsumoto et al. (1986). According to King and Carpenter (personal communication), the Hill coefficient for GABA-induced Cl^- current is 2 in the pleural medial neurons of Aplysia californica. The discrepancy might be accounted for by differences in the species of animals and in the application technique of the agonist.

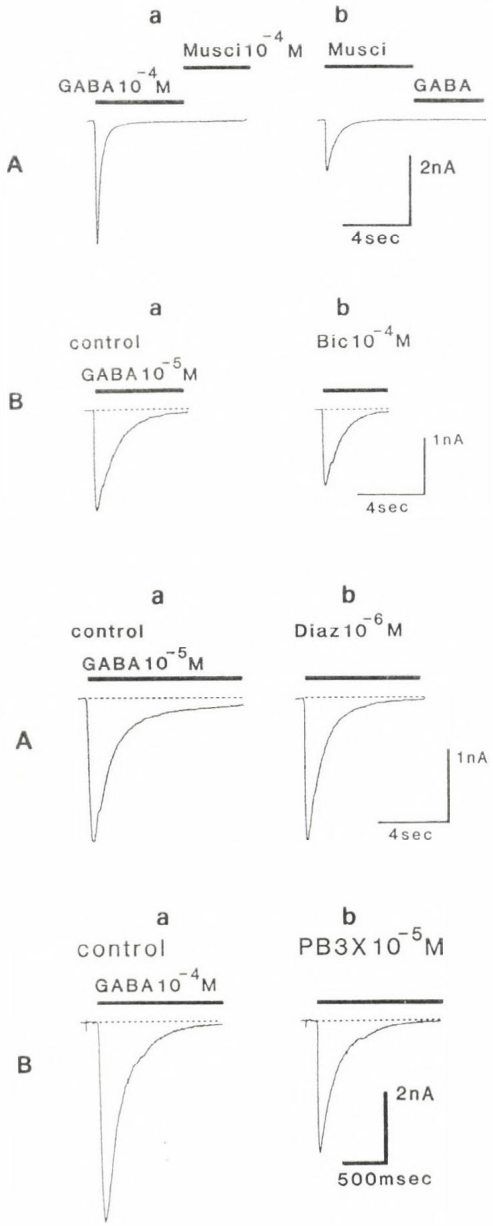

Fig. 4

A: Interaction between GABA-and muscimol-induced Cl^- responses.

B: A slight inhibition of GABA response with bicuculline.

Fig. 5

A: The GABA response with or without diazepam. Diazepam did not enhance the GABA-I_{Cl}.

B: Inhibition of the GABA response by pentobarbital.

In internally perfused dorsal root ganglion cells of the frog, to which GABA was applied by the concentration clamp technique, the activation and desensitization phases of the I_{Cl} were both described by two exponentials. In Aplysia neurons the activation phase was single

exponential, suggesting that GABA activates a single component of the channel. This notion was further supported by the finding that the remaining part of the activatable channel in the progress of desensitization decreased with the same time course as that of desensitization. The desensitization phase and the recovery from desensitization proceeded double exponentially, indicating two states of desensitization. But it is not clear whether the two states are in parallel or in a series.

Pentobarbital (PB) augments the postsynaptic response to iontophoretically-applied GABA and also prolongs the time course of inhibitory postsynaptic potentials in a variety of preparations from vertebrates (Ranson & Barker, 1976; Barker & Ranson, 1978; Huang & Barker, 1980; Nicoll & Wajtowicz, 1980; Willow & Johston, 1983; Segal & Barker, 1984). Similar enhancing actions of PB on the GABA-I_{Cl} were observed in the afferent sensory neurons of the frog, rat and cat having $GABA_A$ receptors. The potentiation of GABA responses was attributed to the increase in the binding affinity of the receptors to GABA in the presence of PB (Ranson & Barker, 1976; Evans, 1979; Connors, 1981; Higashi & Nishi, 1982; Akaike et al., 1985). Recently, we have reported the existence of three different subtypes of $GABA_A$ receptor-Cl^-ionophore complexes in the frog sensory neurons which have different single channel conductances i.e., type 1 (=15 pS), type 2 (=5 pS) and type 3 (=25 pS) and different thresholds of activation for the GABA concentration (Yasui et al., 1985). Interestingly, the facilitatory actions of PB were observed on two types, types 1 and 3 while pentobarbital had no effect on type 2. In the present experiments, when PB was added to the Aplysia neurons by the 'concentration clamp' technique, the agent rather inhibited the GABA-I_{Cl}. In addition, the GABA-I_{Cl} in Aplysia neurons was not modified by diazepam, which enhances GABA binding to the GABA receptor in vertebrate preparations by a mechanism different from that of pentobarbital (Olsen & Snowman, 1982; Hattori et al., 1986). The inhibitory action of bicuculline, a specific GABA blocker was so less potent compared with the actions on various vertebrate neurons. These results suggest the presence of a large difference in the interactions among GABA, barbiturate and benzodiazepine receptors in vertebrate and invertebrate neurons (Fig. 6).

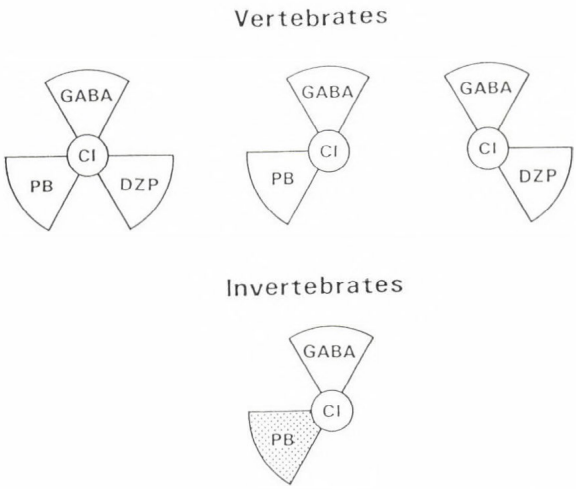

Fig. 6
Schematic illustrations of interactions among $GABA_A$, barbiturate and benzodiazepine receptors in vertebrate and invertebrate neurons. In our recent patch-clamp study using 'inside-out' preparation of frog dorsal root ganglion cells, the majority of recordings showed the interaction among three receptors (upper panel, left), but some (upper panel, middle and right) showed only an interaction between $GABA_A$ and barbiturate or benzodiazepine receptors. In any case, the GABA response was facilitated. Lower panel shows the present results from Aplysia neurons. Note the lack of the benzodiazepine receptor. Pentobarbital interacted inhibitory to the GABA response.

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DISCUSSION

ALKON, D.L.: Is it possible to distinguish desensitization of the receptor response to the agonist from inactivation of the agonist-induced ionic current due to intrinsic biophysical properties (which would have some biochemical basis)?

IKEMOTO, Y.: The desensitization in our experiments includes all processes which contribute to reduce the current overtime despite the continuous presence of a constant concentration of an agonist. It was, therefore, impossible to know how large the contribution of biochemical processes was. Intracellular perfusion of biochemically-active substances may help answer the question.

ERDÉLYI, L.: What do you think about the mechanism of the intracellular Cl maintenance, is it caused by Cl pump or in similar way as it was recently proposed in mammalian preparation by Na,K cotransport?

IKEMOTO, Y.: I have no idea in our preparation. Our neurons were perfused internally, therefore, I think there is no change in the intracellular Cl concentration.

LUKOWIAK, K.: 1. Do all the small cells which you isolate respond to GABA?

2. Have you done a more conventional analysis of GABA on the larger neurons which you cannot use in your present experiments?

IKEMOTO, Y.: 1. Not all the cells responded to GABA. Therefore, I had to discard good cells which had a good action potential, until a GABA-responding cell was obtained.

2. Unfortunately, not. Carpenter published some papers on that matter with iontophoretic application.

MAGAZANIK, L.: What is the cause of disappearance of one component of desensitization kinetics induced by high GABA concentrations? May it be the result of low resolution of method?

IKEMOTO, Y.: Both the fast and slow time constants became smaller with increasing concentrations of GABA. At higher concentrations both values became closer to obscure one component.

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MODIFICATION OF Na-CHANNEL PROPERTIES BY DELTAMETHRIN ON IDENTIFIED NEURONS OF HELIX POMATIA L.

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The primary target site of pyrethroid insecticides is sodium channels of excitable membranes. Binding to the Na-channel molecule from outside, pyrethroids greatly prolong the open state of individual sodium channels (Yamamoto et al. 1983, Chinn and Narahashi 1986). Due to this, at the macroscopic current levels the Na inward current is prolonged because Na-channels do not or only very slowly inactivate.

The single channel data (Chinn and Narahashi 1986), however, showed that the closed or inactivated states were also stabilized in the presence of pyrethroid. The data are consistent with the hypothesis that some pyrethroids reduce the rate at which transitions occur between closed, open and inactivated states of the ionic channel. Lund and Narahashi (1982) and Lund (1984) proposed a kinetic model, according to which in the pyrethroid-treated neuronal membrane, channels exist simultaneously both with normal and modified kinetic states.

The present experiments were performed in order to study the action of deltamethrin on molluscan neuronal membrane, since data on this group of animals are scarce.

MATERIAL AND METHODS

Experiments were performed on LPa1 and RPa3 neurons of the land snail Helix pomatia L. Both are giant neurons in which the raising phase of action potential is mainly Na^+-dependent. The membrane potential and the membrane currents were recorded

using a two-microelectrode voltage-clamp technique (Véró 1974). Low resistance 1.5-5.0 M microelectrodes were used and filled with 2.5 M KCl. Current electrodes were covered with silver paint and grounded in order to minimize the coupling capacitance between the two electrodes. The tip of the microelectrodes was covered by varnish to be isolated from the external solution. In order to eliminate outward current components and calcium inward current a physiological saline with the following composition was used (mM): NaCl - 90, KCl - 4, $CaCl_2$ - 1.8, $MgCl_2$ - 5, TEAC - 30, 4-aminopyridine (4AP) - 5, and Tris-Cl - 10, pH=7.4. The stock solution of deltamethrin was made in dimethylsulfoxide (DMSO) at concentrations of $5x10^{-3}$ M daily. This stock solution was then diluted in the above solutions which formed a milky suspension. Therefore, the actual concentration of deltamethrin in the solution was unknown. All experiments were carried out at a constant temperature of $18^{o}C$.

RESULTS

In Fig. 1 spontaneous action potentials can be seen recorded in control (A) and pyrethroid-containing solutions (B). The insecticide depolarized the neuron, decreased the AP amplitude and increased the repolarization process. The depolarizing after-potential was increased both in time and amplitude. In Fig. 2 the characteristic effect of deltamethrin can be seen at different membrane voltages. There is a 5-20% decrease or no change at all in the peak values of the Na-current as compared to the controls. On the contrary, the relaxing phase of the Na-current was substantially slowed down. Current-voltage relationship before and after deltamethrin treatment is presented in Fig. 3. Curves I-V were obtained after correction for leakage current component.

In Fig. 3 the peak values in the control solution, while after deltamethrin treatment the peak and current values at the end of the 40 ms depolarizations are plotted. The reversal potential for sodium current (40-50 mV) was the same in the control and the poisoned neuronal membrane. Figure 4 shows the

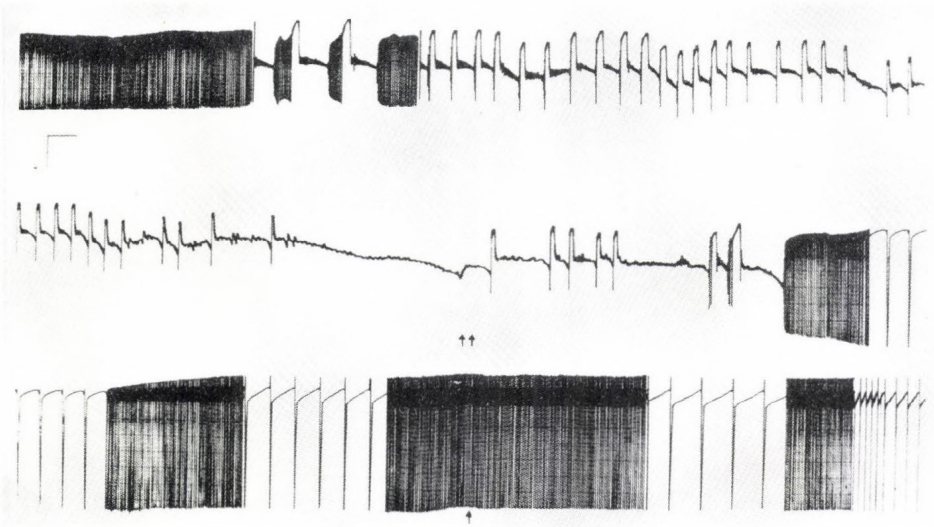

Fig. 1. Effect of deltamethrin on the spontaneously generated action potential of LPa1 neuron: (↓) moment of pyrethroid application, (↓↓) starting of the washout procedure.

steady-state level of activation and inactivation variables as a function of the membrane potential. Practically no significant change in activation was observed, while the inactivation curve was shifted to the right along the voltage axis by 10-15 mV. These data indicate that channels which undergo inactivation are of different voltage dependence than those in the control solution. The time constants of inactivation were determined from semilogarithmic plots of the current decaying phase.

It was found that under control circumstances channels inactivate mainly along one exponential and have a biexponential relaxing phase in a very low percentage (Fig. 5). Following deltamethrin treatment, however, in all experiments the inactivating phase of the Na-current was biexponential, characterized by fast (τ_1=5-10 ms) and slow (τ_2=35-40 ms) components.

In Fig. 5 the voltage dependence of inactivation time constants is shown. The effect of deltamethrin on the membrane Na-channels revealed time dependence because by the passing of time the number of slowly inactivating (or modified) sodium channels was increased. The tail current measured upon repolar-

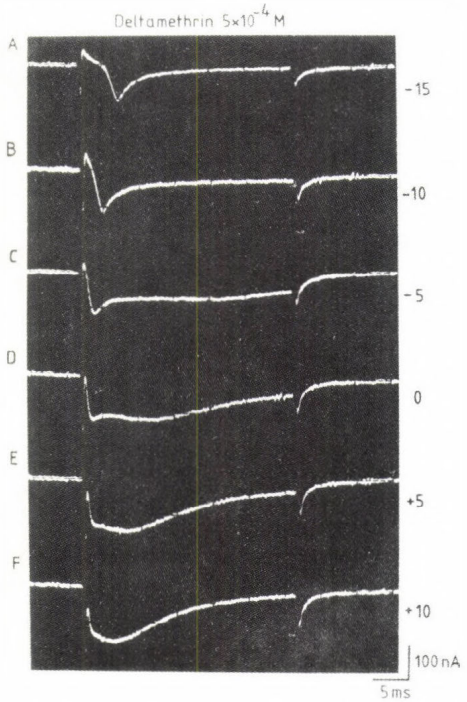

Fig. 2. Effect of deltamethrin on the Na-inward currents. Numbers on the right side represent voltage level to which the membrane was depolarized. HP = -50 mV, cell RPa1.

ization was very small in the control saline, however, in the presence of deltamethrin a large tail current was recorded upon repolarization. This current represents channels which were open at the end of depolarization, therefore mainly pyrethroid modified the channels. The potential (A) and time (B) dependence of the tail current is shown in Fig. 6. The semilogarithmic plot of the tail currents revealed two components, a fast and a slow (Fig. 7). The fast and the slow components are in good agreement with the time constants observed during inactivation of the Na-current (Fig. 5). In order to elucidate whether deltamethrin-modified channels inactivate to the normal or modified inactivated state, the recovery process from inactivation was studied. The membrane was conditioned for 40 ms at 0 mV, then allowed to recover at -50 mV for increasing periods of time, and then at 0 mV peak currents were recorded

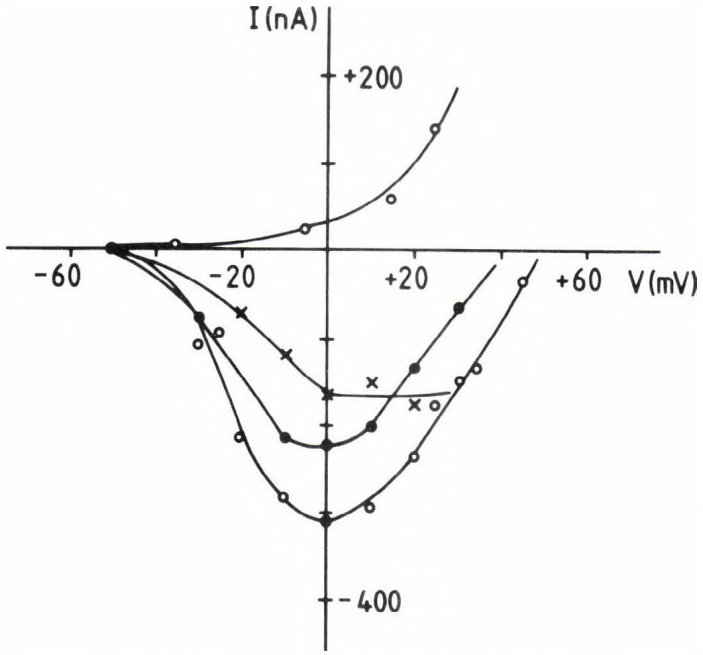

Fig. 3. Potential dependence of the peak values of inward current in control (o) and poisoned membrane (●). (x) Current values measured at the end of 40 ms pulse.

(Fig. 8). Recovery occurred with a single exponential time course with a time constant of 8 ms both in the control and deltamethrin-treated membrane.

DISCUSSION

Deltamethrin had almost no effect on the peak values or decreased the Na-inward current; at the same time a significant prolongation of inactivation was observed in snail neurons. Also the tail current amplitude was increased. Kinetic analysis of the Na-current revealed that the activation variable was not changed while the inactivation parameter showed different voltage dependence in the presence of deltamethrin. The kinetic variable was shifted to the depolarizing direction along the voltage axis, which is different from the results obtained by other pyrethroids (Lund and Narahashi 1981a,b).

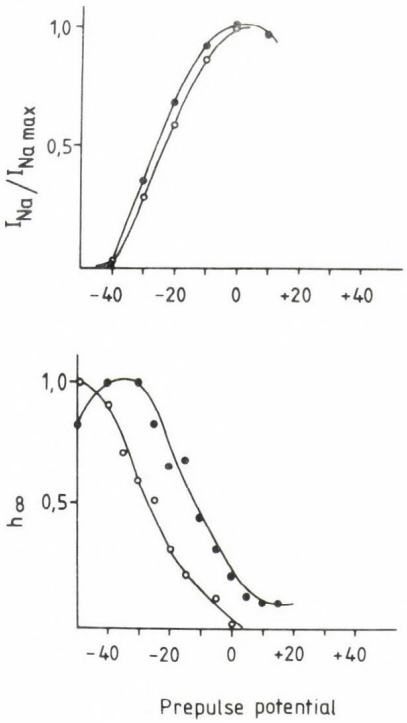

Fig. 4

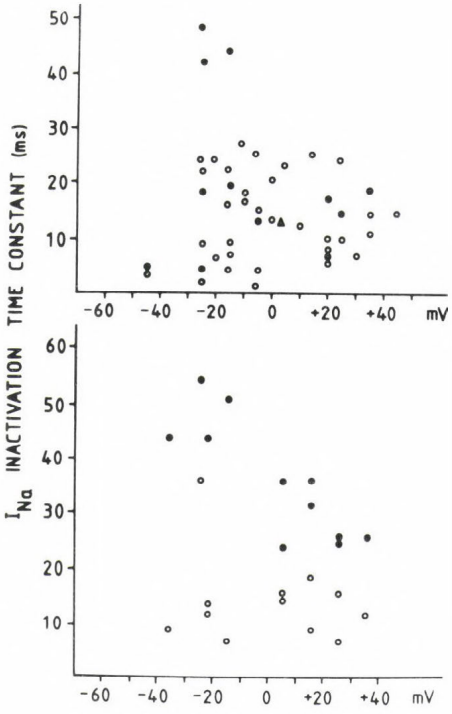

Fig. 5

Fig. 4. Voltage dependence of activation and inactivation parameters in control (o) and deltamethrin-treated neurons (●).

Fig. 5. Semilogarithmic plot of the inactivating phase of the Na current revealed the presence of two time constants: a fast (τ_1) and a slow (τ_2) one. Upper panel: potential dependence of τ_1 (o) and τ_2 (●) in control saline. Lower panel: the same after deltamethrin treatment.

According to Chinn and Narahashi (1986), the simplified mechanism of pyrethroid action could be that the drug decreases the transition rates from one kinetic state to another. It was therefore suggested that all non-modified and modified kinetic states occur simultaneously in the drug-treated membrane. Recently, Lund (1984) proposed a kinetic model of Na-channel function in a membrane poisoned with tetramethrin. According to this model, in the pyrethroid treated membrane all channels activate normally. When the channel reaches the open state it can either inactivate normally or, interacting with the tetra-

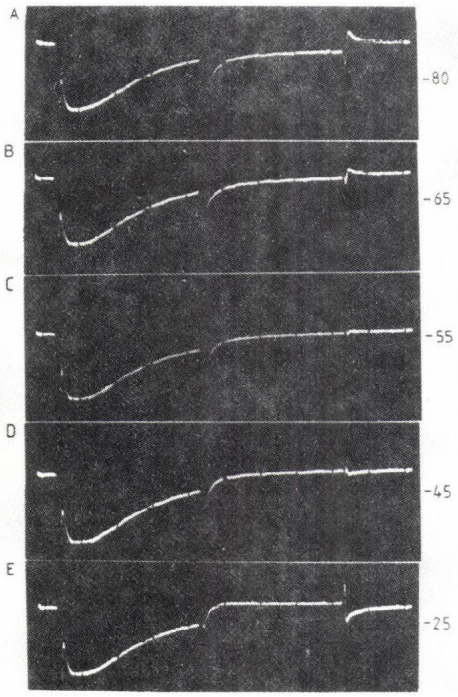

Fig. 6. Voltage dependence of the tail current. Numbers on the right side show the voltage level after the pulse. HP = -50 mV, test pulse +50 mV. Duration of the test pulse was 40 ms.

methrin molecule, can enter the modified open state, which inactivates slowly to a modified inactivated state.

However, our recovery experiments from the inactivated state suggested that both modified and non-modified channels might inactivate into the same state.

Therefore, a kinetic model can be proposed at least for the deltamethrin action, in which only open and closed states are modified (Narahashi 1985). The modified open state can account for the second open state of the Na-channel (Nagy et al. 1983, Nagy 1986). From these two open states the channels inactivate with different time constants, which were found to be similar in both treated and untreated neurons. However, the probability of their entering into a second open state was higher in the presence of deltamethrin.

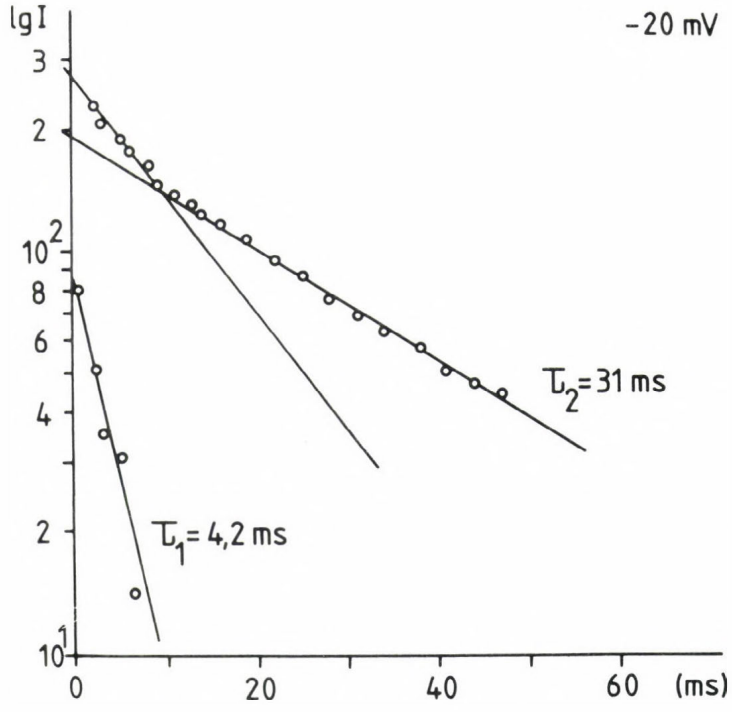

Fig. 7. Semilogarithmic plot of the tail current. Time constants are similar to the inactivation time constants of inward current. HP = -50 mV, the potential shown in the upper right represents the level of depolarization.

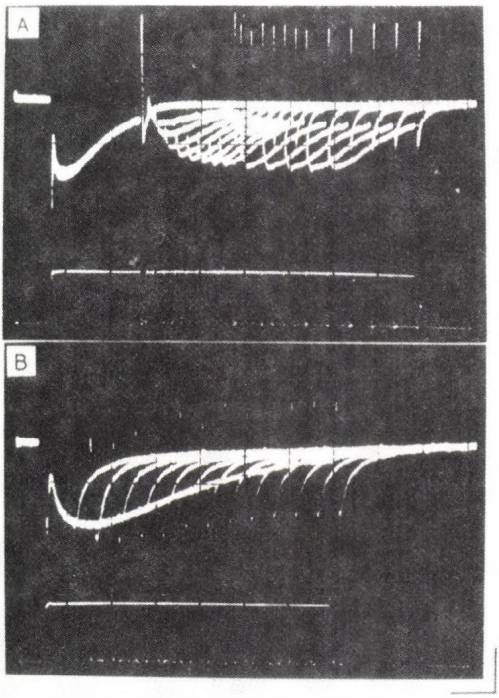

Fig. 8. A. Recovery from inactivation observed along one exponential with time constant of 8 ms. B. Tail currents recorded after depolarizing pulses of different duration. Calibration 10 ms and 100 nA.

CONCLUSIONS

1. It was found that snail neurons are suitable for studies of pyrethroid effects.

2. Deltamethrin in 5×10^{-5} M concentration prolonged markedly the inactivation of the Na-current, and increased and prolonged the tail current.

3. Changes in the properties of the Na-current resulted in the appearance of a fraction of insecticide-modified channels.

4. A kinetic model is proposed, according to which deltamethrin interacts with the second open state of the Na-channel which inactivates much slower than the normal ones.

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DISCUSSION

LEAKE, L.: I am worried by the concentration of 5×10^{-5} M deltamethrin you are using as it is much higher than the concentration of approx. nmolar required for the toxicological response. We have found that Lymnaea is some 1000x more sensitive to pyrethroids than Helix. Have you tried using Lymnaea for your studies?

KISS, T.: I have worked only on Helix neurons, and have not tried Lymnaea. However, if Lymnaea neurons are so sensitive to deltamethrin it would be worthwhile to repeat the experiments on Lymnaea neurons, too. As regards the concentration: on many preparations such concentrations were used since these gave a very clear change in Na-current.

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EFFECT OF LOW CONCENTRATION OF GABA ON THE ACh SENSITIVITY OF SNAIL NEURONS

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Previously it has been shown that the effectivity of transmitter action on the membrane depends to a certain degree on the activity of membrane enzymes, especially on Na,K-ATPase and adenylate cyclase. As it was established, inactivation of the Na,K-pump leads, on the one hand, to the increase of functionally active membrane receptors (through the pump induced cell volume changes) and, on the other hand, to the decrease of transmitter-receptor affinity as a result of membrane phosphorylation (Ayrapetyan et al., 1979, 1985).

We proposed that besides the effects on membrane ionic permeability, transmitters induce direct metabolic changes in the cell, too. However, it is difficult to separate the transmitter-induced permeability changes from the metabolic effects. In order to elucidate the metabolic significance of ACh and GABA effects on the cell the influence of low concentrations of these transmitters (i.e. the concentrations at which membrane potential and permeability are hardly affected) on the Na,K-pump activity, and Na:Ca exchange was studied. We also examined the effect of low concentration of GABA (10^{-8} M) on the ACh sensitivity of the postsynaptic membrane.

RESULTS

In order to study the mechanism of ACh and GABA on the Na,K-pump activity, upon application of these transmitters the Na ion efflux from the cells was investigated. Figure 1 shows the

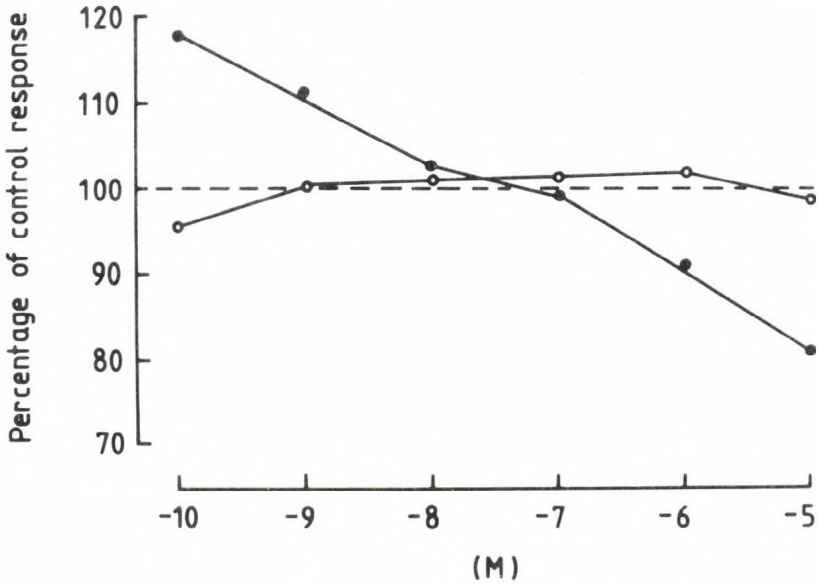

Fig. 1. Effect of ACh (●) and GABA (o) on the ^{22}Na efflux from the cells in normal physiological solutions. Abscissa - log concentration of transmitters; ordinate - ACh and GABA induced efflux changes as compared with control efflux.

dose-dependent action of ACh and GABA on Na-efflux from the cells. As it is shown, the low ACh concentrations led to activation, while the increased concentration to the inhibition of Na efflux from the cells. However, GABA in concentrations of 10^{-10}-10^{-5} M had no dose-dependent effect on the efflux of Na ions from the cells.

It is known that Na efflux in snail neurons is realized by two mechanisms: Na,K-pump and Na:Ca exchange (Ayrapetyan et al., 1984). The Na,K-pump, the functional molecule of which is Na,K-ATPase, can be totally blocked by 10^{-4} M ouabain (Ayrapetyan et al., 1984). To elucidate the effect of neurotransmitters on Na:Ca exchange a series of experiments were performed in K-free, 10^{-4} M ouabain-containing solution. Figure 2 shows that in this medium ACh and GABA had a dose dependent activating effect on the efflux of Na ions from the cells. It is worthy to note that both curves had saturating character and reached their maximum at 10^{-11} M and 10^{-10} M concentrations for

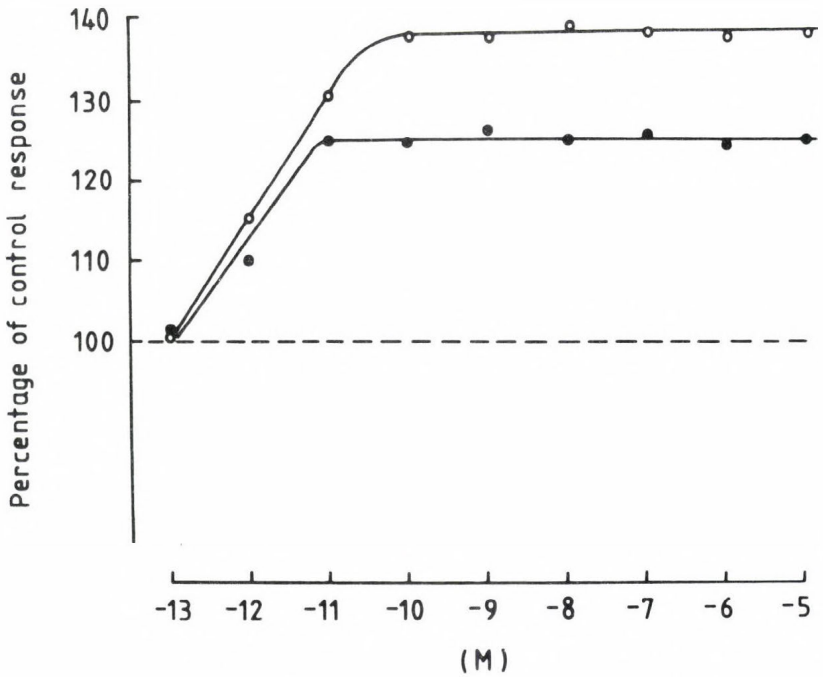

Fig. 2. Effect of ACh (●) and GABA (o) on the ^{22}Na efflux from the cells in K-free, 10^{-4} M ouabain-containing solution. Abscissa - log concentration of transmitters; ordinate - ACh and GABA induced changes of ^{22}Na efflux from the cells as compared with the efflux in K-free, 10^{-4} M ouabain-containing solution.

ACh and GABA, respectively. ACh increased the Na efflux from the cell by 25% while GABA by 38%.

The close correlation between Na:Ca exchange and intracellular cAMP level was shown by Bitter et al. (1976) on the barnacle muscle fibres. The increase of cAMP level led to the enhancement of Na:Ca exchange rate. Since adenylate cyclase is one of the main enzymes in the cell defining the level of cAMP synthesis, in the next series of our experiments the effect of ACh and GABA on Na:Ca exchange was studied in conditions when the adenylate cyclase system was stimulated by NaF (10^{-2} M). The experiments were carried out in K-free, ouabain-containing medium. Figure 3 shows that NaF stimulates the Na:Ca exchange by 54% as compared to the control. These data suggest that

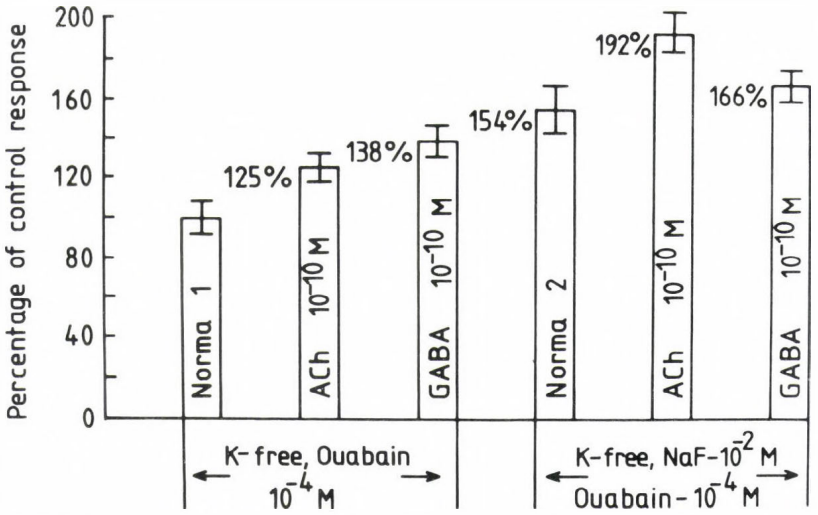

Fig. 3. Effect of ACh and GABA on Na:Ca exchange in K-free, 10^{-4} M ouabain (norma 1) solution and in the presence of NaF (norma 2). Ordinate - change of ^{22}Na ion efflux from the cells as compared to the norma 1.

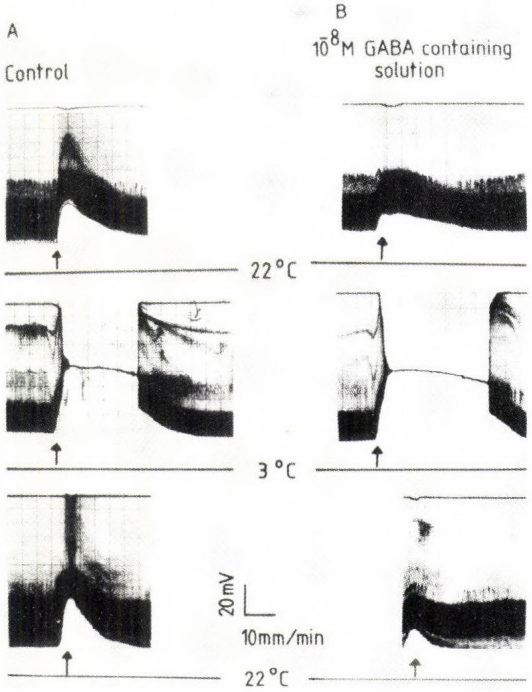

Fig. 4. ACh-induced depolarization of postsynaptic membrane potential in the absence (A) and presence (B) of 10^{-8} M GABA.

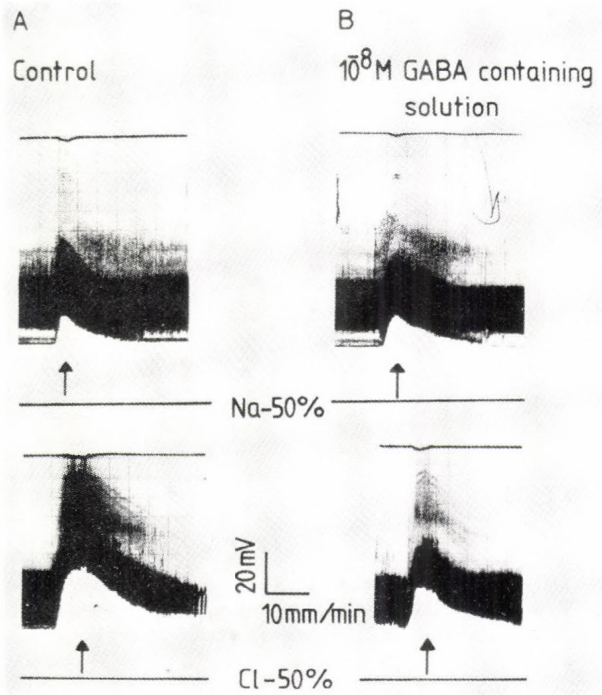

Fig. 5. ACh-induced depolarization of postsynaptic membrane potential when Na and Cl ion concentrations of the extracellular solution were decreased by 50% in the absence (A) and presence (B) of 10^{-8} M GABA.

activation of adenylate cyclase leads to the increase of Na:Ca exchange in snail neurons. It should be noted that the activating effect of ACh on Na:Ca exchange does not depend on the presence of NaF in the bathing medium. However, the activating effect of GABA on Na:Ca exchange was significantly decreased in the presence of NaF.

GABA in a concentration of 10^{-8} M had no significant effect on the membrane permeability. But in this concentration the activity of Na,K-pump was inhibited (Fig. 1) and the Na:Ca exchange was stimulated (Fig. 2). These processes appeared to be saturated at this concentration of GABA.

Two types of neurons were identified according to ACh response in GABA (10^{-8} M)-containing solutions. In one type of the neurons, where reversal potential of ACh-induced current was around -20 to -30 mV, GABA had no effect on ACh-response.

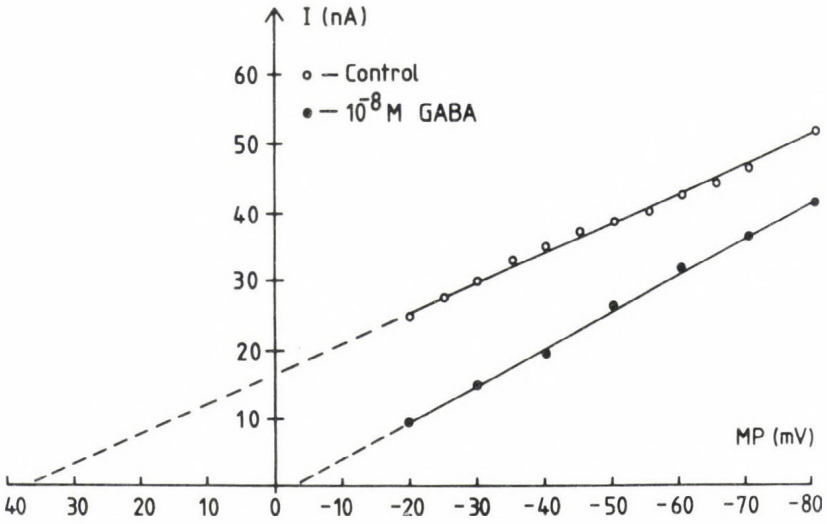

Fig. 6. ACh-induced voltage-current characteristics of postsynaptic membrane in the absence (o) and presence (•) of 10^{-8} M GABA. Abscissa - membrane potential; ordinate - transmitter mediated inward currents.

In case of the other type of neurons, as presented in Fig. 4, GABA decreased the ACh-induced depolarization at room temperature ($22^{o}C$). At $3^{o}C$, however, the decreasing effect of GABA on the ACh response was not present. Returning to room temperature, the depressing effect of GABA on the ACh-induced depolarization was found even more effective.

In order to find out the ionic mechanism of the inhibitory effect of GABA on the ACh-induced current we changed the Na and Cl concentrations of the external solution. As can be seen in Fig. 5 (upper part), GABA had no effect on the ACh response in solution containing 40 mM instead of 80 mM NaCl.

Decreasing the Cl ion concentration in the external solution the inhibitory effect of GABA on ACh response was unchanged as compared to the control (Fig. 5, lower part).

Under voltage-clamp experimental condition (Fig. 6) the reversal potential of ACh-induced current in control solution was found to be around +30 to +40 mV, and in the presence of GABA it was observed to shift to the values of 0 to -15 mV. There-

fore, it seemed probable that in GABA containing solution the Na-dependent ACh-induced ionic currents were modified.

DISCUSSION

It is known that in the CNS of mollusc the effective dose of synaptic transmitters generating the transmembrane currents is in the range of 10^{-6}-10^{-4} M. To determine the metabolic effect of ACh and GABA action low doses of these transmitters were used in this work.

The antigradient Na ion efflux from the cell consists of ouabain-sensitive and ouabain-insensitive components (Ayrapetyan et al. 1984). The ouabain-sensitive component is the Na,K-pump, while the insensitive one represents a second transport system which is mainly defined by Na:Ca exchange in the neuronal membrane.

Our data showed that in normal physiological solution ACh activated or inactivated the Na efflux depending on its concentration, while GABA had no essential effect on Na efflux. However, both transmitters had activating effect on Na:Ca exchange when the ouabain-sensitive component was blocked. From these data it can be suggested that both transmitters inactivated the Na,K-pump, while Na:Ca exchange was activated conversely.

It is interesting to note that ACh and GABA activated the Na:Ca exchange in a dose-dependent manner which saturated at low concentrations (10^{-11} M and 10^{-10} M of ACh and GABA, respectively). The activating effect of GABA on Na:Ca exchange was stronger as compared to the effect of ACh (see Fig. 2).

Previously it had been shown that GABA increased the intracellular level of cAMP and cGMP (Dadalian et al. 1987). The GABA-induced cAMP accumulation in the presence and absence of ouabain and stimulation of the Na:Ca exchange allow us to suggest that GABA has an effect via activation of adenylate cyclase activity. This suggestion is supported by experiments in which NaF, an adenylate cyclase activator, was used. In these experiments the activating effect of GABA on Na:Ca

exchange (which is facilitated by increase of intracellular cAMP level) disappeared.

Similar results were obtained by Bittar et al. (1976) on the barnacle muscle fibre where raising the intracellular level of cyclic AMP resulted in an increase of Na:Ca exchange.

Low doses of transmitters increased intracellular Ca concentration as a result of increase in permeability of cAMP-dependent Ca-channels (Kostyuk et al. 1983) and Na:Ca exchange.

The activation of Na:Ca exchange by low dose of transmitters (increase of intracellular Ca concentration) and also the enhancement of cGMP level allow us to conclude on the physiological significance of low dose transmitter-induced modulation of different cell activities.

We have shown that low doses of ACh and GABA increased the intracellular cAMP level which is the second messenger for regulation of different neuronal functions.

In visceral primary afferent neurons of the rabbit Higashi et al. (1987) had shown the inhibition of Na,K-pump due to an increase in intracellular Ca ion concentration. On the other hand, Arvanov and Ayrapetyan (1980) found that ouabain, the specific inhibitor of Na,K-pump, decreased the ACh-induced current in snail neurons. It was also found that inhibition of the Na,K-pump led to an increase in the intracellular level of cAMP (Dadalian et al. 1987). It was proposed that protein phosphorylation, which plays an important role in the regulation of metabolic pathways (Krebs and Beavo 1979) and which has been suggested to be involved in the regulation of various ion channels in neurons (Levitan 1985), may play a role in the slow desensitization step of the receptor protein. In fact, postsynaptic membranes from Torpedo electric organ, enriched in ACh receptors, contain at least three different endogenous protein kinases: a cyclic AMP-dependent protein kinase (Huganir et al. 1983a), a Ca-phospholipid-dependent protein kinase, protein kinase C (Huganir et al. 1983b), and tyrosine specific protein kinase (Huganir et al. 1984). In addition, these endogenous protein kinases phosphorylate the ACh receptor at different specific sites, which are located most likely on the intracellular domain of the receptor (Huganir et al. 1984).

Earlier we have also shown that the affinity of membrane receptors to their transmitters is regulated by the degree of membrane protein phosphorylation (Ayrapetyan et al. 1985). Thus, it is suggested that transmitters themselves (ACh and GABA) could regulate the affinity of their own as well as of other transmitter receptors, stimulating membrane protein phosphorylation via cAMP or Ca-phospholipid-dependent protein kinase activity. From these data we suggested that the inhibitory effect of 10^{-8} M GABA on the ACh-induced depolarization goes through intermediate reaction steps in the cell, because this effect was abolished at $3^{\circ}C$.

It is of importance that the inhibitory effect of GABA on ACh evoked responses was found to be dependent on the intracellular concentration of Na ions. The increasing intracellular concentration of Na ions was due to the decrease of electrochemical gradient and to the increase in the rate of Na:Ca exchange, especially in the presence of 10^{-8} M of GABA. On the basis of these results it is assumable that the Na:Ca exchange may play a role in the effect of GABA on ACh evoked depolarization. Therefore, the conclusion could be drawn that the inhibitory effect of 10^{-8} M GABA is realized not on the ACh-receptor molecule itself, but rather through intracellular biochemical machinery.

It is known that the release of transmitters and hormones depends on intracellular Ca ion concentration (Miledi 1973) and that transmitter interaction may take place at the level of their releasing processes by Na:Ca exchange modulation.

On the other hand, the gradual increase of the transmitter concentration in bathing solution decreases the response of the postsynaptic membrane. cAMP increase induced by low doses of ACh and GABA can stimulate the activity of both cAMP-dependent and Ca-phospholipid-dependent protein kinases (through the increase of Ca concentration), causing membrane protein phosphorylation. The desensitization of postsynaptic membrane receptors is presumedly based upon this mechanism.

CONCLUSIONS

1. Both ACh and GABA influence not only the permeability of the postsynaptic membrane, but also inactivate the Na,K-pump and activate the Na:Ca exchange in the snail neurons. However, the latter effect can be detected at low ACh and GABA concentrations (10^{-11} and 10^{-10} M, respectively).

2. A 10^{-8} M concentration of GABA decreased the ACh sensitivity of the postsynaptic membrane through intracellular biochemical mechanism.

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cAMP INCREASES EXCITABILITY IN GROWTH HORMONE PRODUCING NEURONS BY ENHANCEMENT OF Ca-CURRENT

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SUMMARY

The membrane excitability of the peptidergic growth hormone cells in the pond snail *Lymnaea stagnalis* shows considerable variation with time, that appears to be subject to cAMP dependent regulation. An increase in the level of intracellular cAMP increases the membrane excitability, in that both spiking threshold and the rate of accommodation strongly decrease. In voltage clamp recordings, bath application of 8-CPT-cAMP, a membrane permeable analog of cAMP, increases the peak inward current, whereas the fast K-current is not affected and the steady state outward current shows slight, but variable changes. The up to twofold increase in inward current persists in Cs-loaded cells. The effects of cAMP on spiking and inward current are blocked by Co and Ni, but not by TTX, suggesting that cAMP affects the Ca-current. The effects of cAMP were, therefore, further investigated in whole cell voltage clamp recordings of isolated GHCs. Under these conditions, 8-CPT-cAMP caused an increase of the isolated Ca-current (which was completely blocked by 10^{-3} M Ni) and a 10 mV shift of its activation range towards more negative potentials. Also, the wash out of Ca channels which normally occurs in perfused cells, was strongly decreased by 8-CPT-cAMP.

It is concluded that a rise in the level of intracellular cAMP enhances the voltage dependent Ca-current in GHCs, thereby enhancing membrane excitability. The increase in Ca-current causes a shift from predominantly Na-dependent spikes, which rapidly accommodate and have a high threshold, to predominantly Ca-dependent spikes, which have a lower threshold, and, due to much slower and incomplete inactivation of the Ca-current, show less accommodation.

INTRODUCTION

The membrane excitability of neuroendocrine cells shows considerable variation with time. Consequently, their electrical behaviour can be described in terms of different states of excitability (Kits, 1980; Kaczmarek *et al.*, 1982; de Vlieger & Lodder, 1985). The regulation

of these excitability states appears to involve second messengers that mediate long term modulation of ionic currents (see Levitan, 1985; Kaczmarek, 1987). Profound effects of second messengers on the electrical behaviour of neuroendocrine cells have been demonstrated in the bag cells of *Aplysia* and the Caudo-Dorsal Cells of *Lymnaea*, where cAMP evokes long lasting discharges, resembling the normal firing patterns of these cells (Kaczmarek *et al.*, 1978; Kaczmarek & Strumwasser, 1981; Moed *et al.*, 1987). Kaczmarek and co-workers showed that this involves cAMP mediated inactivation of the fast K-current (Strong, 1984) and partial suppression of delayed K-currents (Kaczmarek *et al.*, 1986). In bag cells, the Ca-current is enhanced by protein kinase C (DeRiemer *et al.*, 1985). In *Helix* neurones enhancement of the Ca-current by cGMP dependent protein kinase has been reported (Paupardin-Tritsch *et al.*, 1986). Modulation of Ca-currents has received much attention in cardiac cells, where analogs of cAMP enhance the Ca-current (Reuter, 1983) and cGMP antagonizes this effect (Hartzell & Fischmeister, 1986). In dorsal root ganglion cells the Ca-current is reported to be decreased by activators of protein kinase C (Rane & Dunlap, 1986) and by analogs of GTP (Scott & Dolphin, 1986).

The present paper concerns cAMP dependent regulation of membrane excitability in the peptidergic growth hormone cells (GHCs) of the pond snail *Lymnaea stagnalis*. The GHCs are situated in 4 clusters in the cerebral ganglia and produce a number of peptides, amongst them an insulin like factor, that are thought to account for the stimulating effect on growth (Geraerts, 1976; Ebberink *et al.*, 1987). In GHCs excitability is greatly enhanced by an increase in intracellular cAMP and the cells shift from a predominantly Na-dependent electrical behaviour to a mainly Ca-dependent state. The mechanism of this effect involves a marked increase in the voltage dependent Ca-current. In GHCs cAMP dependent regulation of membrane excitability may involve the D-1 and D-2-like dopamine receptors previously characterized in these cells (Stoof *et al.*, 1984).

MATERIALS AND METHODS

Adult, laboratory bred *Lymnaea stagnalis* were used. The preparation consisted of the isolated central nervous system, including the buccal ganglia. For whole cell clamp recordings isolated GHCs in primary culture were used, always within 8 hrs after isolation. Isolation was done mechanically, after a 30 min treatment with 0.2% trypsine (37°C).

Standard Hepes buffered saline had the following composition (in mM): 30 NaCl, 1.7 KCl, 5 $NaHCO_3$, 10 $NaCH_3SO_4$, 1.5 $MgCL_2$, 4 $CaCl_2$, 10 Hepes; pH 7.6. Isolated cells were kept in standard saline to which Eagle's Minimal Essential Amino Acids and Vitamins, antibiotics and glucose were added. For isolation of Ca-currents under whole cell voltage clamp, a bath saline containing (in mM) 10 $BaCl_2$, 35 TEACl, 10 Hepes, 7 CsOH and 3×10^{-5}

M TTX (pH 7.6) was used; the pipette solution contained 50 TEACl, 10 Hepes, 7 CsOH (pH 7.6).

Intracellular recordings were made using conventional electrophysiological equipment. Two electrode voltage clamp recordings were made using a Dagan 8500 amplifier. Whole cell voltage clamp recordings were made with a Dagan 8900 patch clamp amplifier (feedback resistance 0.1 GOhm; electrode resistance 1-4 MOhm; seal resistance >1 GOhm, series resistance 2-6 MOhm). Whole cell clamp recordings were only made when the fast K-current was completely removed by the TEA-saline, usually within 20 min after establishing the whole cell clamp condition, indicating equilibration of pipette and cell content. Under standard conditions the washout of Ca-current amounted to about 50% after 1 hr. Control and experimental recordings were therefore made within 45 min after establishing the whole cell clamp condition. Successive step depolarizations were applied at 30 s intervals. IV-protocols were generated by a CED1401 plus Tandon PC system. Recordings were stored on FM tape (TEAC XR-310) and analysed off line. For analysis a transient recorder (Datalab DL902) and a HP1000 computer or a CED1401 and a Tandon PC were used.

RESULTS AND DISCUSSION

The GHCs in *Lymnaea stagnalis* are usually silent when recorded in the freshly prepared isolated central nervous system. Fig. 1 shows that high threshold action potentials are evoked by depolarization (current injection of 2 nA), the response accommodating very rapidly. The action potentials are mainly Na-dependent since they are abolished in Na-free saline and by TTX (3x 10^{-5} M), whereas Ca-blockers, such as 10^{-3} Co, do not block the spikes (cf. de Vlieger & Lodder, 1983, 1985). It appeared that several experimental treatments may increase the excitability of the cells (de Vlieger & Lodder, 1985). Short afterdischarges upon electrical stimulation or even spontaneous spiking can be induced by application of 4-amino-pyridine to block fast K-channels, by substitution of Ca by Ba, and by short term culture of the cells (>24 hrs). Finally, an increase in the level of intracellular cAMP increased the membrane excitability as illustrated in Fig. 2. The effect of increasing intracellular cAMP consists of a decrease in

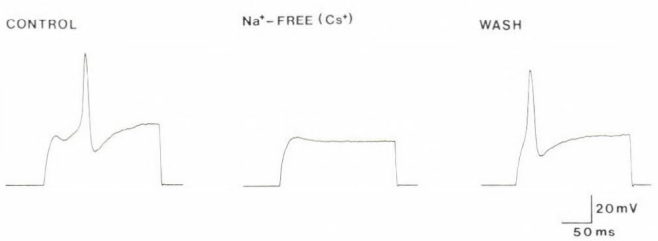

Fig. 1.GHC response to depolarization by 2 nA current injection. The cell has a high threshold and accommodates fast. The spike is blocked in Na-free saline (Na replaced by Cs).

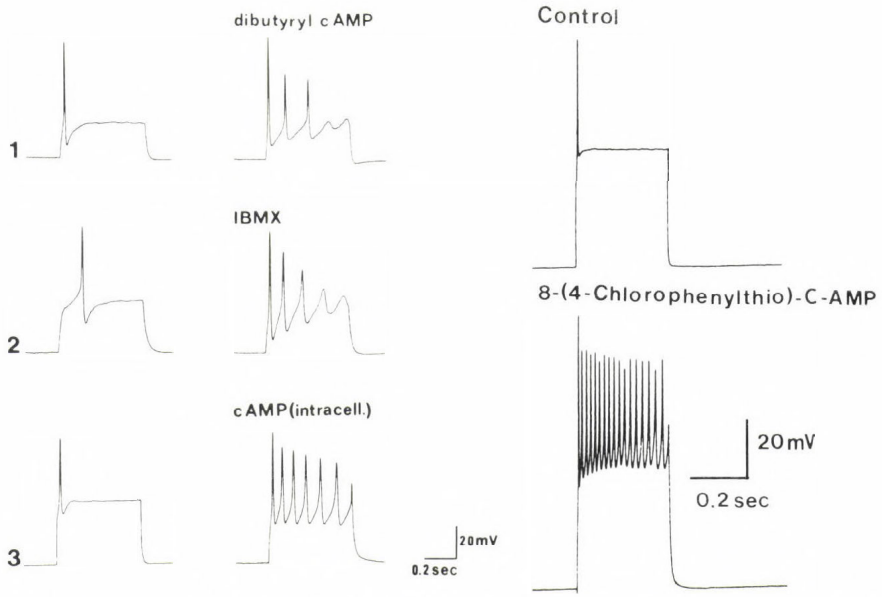

Fig. 2.GHC responses before and after increasing the intracellular level of cAMP (cAMP increased by methods as indicated), showing that after the cAMP treatment the spiking threshold is decreased and the cells only slowly accommodate.

both spiking threshold and the rate of accommodation and is brought about by any of several ways to achieve the rise in cAMP content, viz. injection of cAMP, application of membrane permeable analogs such as 8-CPT-cAMP or dibutyryl-cAMP, or application of IBMX to inhibit fosfodiesterase or forskolin to activate adenylate cylcase. The ionic mechanism of the spikes, as probed by the action of Na- and Ca-blockers, is changed by the cAMP treatment. Fig. 3 shows that after increasing intracellular cAMP, TTX no longer abolishes spiking, but only slightly affects the number and height of the spikes evoked by depolarizing pulses. The Ca-blockers Co (not shown) and Ni, however, almost completely block spiking, indicating that the spikes are now predominantly Ca-dependent.

To investigate the mechanism underlying the changes in excitability, the effect of 10^{-4} M 8-CPT-cAMP on voltage dependent currents was investigated by means of two electrode voltage clamp recordings. Under control conditions (holding potential -60 mV, standard saline, no blockers) 8-CPT-cAMP caused a consistent increase in inward current: the maximum peak inward current was doubled in size and the peak activation shifted by about 10 mV in the negative direction (Fig. 4). 8-CPT-cAMP did not cause a change in the fast K-current (not shown), whereas the effect on the late outward current was small and variable. The increase in inward current persisted when TTX was added to the bath. We therefore designed experiments to study the effect of 8-CPT-cAMP on the isolated Ca-current. To isolate the Ca-

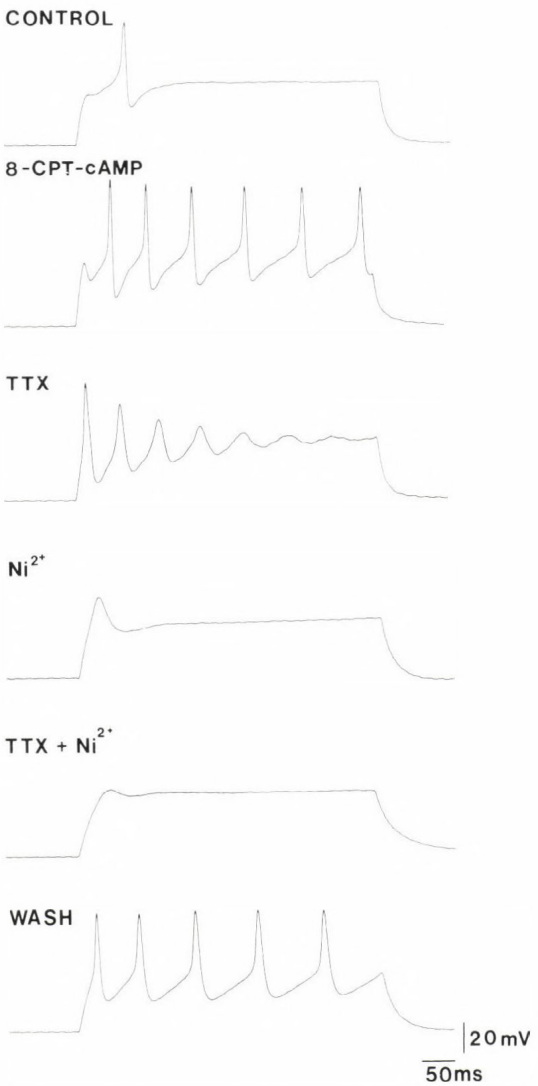

Fig. 3. Effect of Na and Ca-blockers on cAMP treated GHCs. TTX (3×10^{-5} M) only slightly affects the spikes, whereas Ni (10^{-3} M) almost completely blocks spiking. Both treatments are reversible.

current GHCs were loaded with Cs-saline (60 mM CsCl, 9.5 mM $MgCl_2$, 10 mM Hepes, 40 mM sucrose) by permeabilizing the cells with nystatin (50 mg/l; 30 min). Subsequently the cells were washed for another 30 min using standard saline with Cs replacing K and Ba replacing Ca, after which 3×10^{-5} M TTX was added and recordings were made. Under these

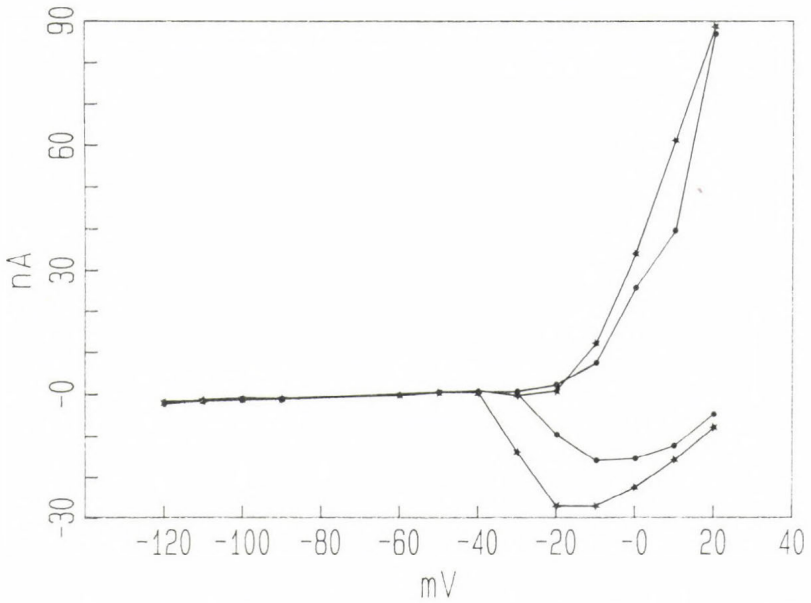

Fig. 4.IV-relationship of peak inward and late outward current obtained by two electrode voltage clamp recordings before (●) and after (✱) application of 10^{-4} M 8-CPT-cAMP. cAMP treatment increases the peak inward current. (Standard saline; HP -60 mV.)

circumstances the inward current was completely blocked by Co. Addition of 8-CPT-cAMP caused a marked increase in peak inward current (Fig. 5), that also was completely blocked by Co. These results suggested that cAMP enhances the Ca-current. However, outward currents were still seen at command potentials of >10 mV, showing that no complete isolation of the Ca-current was achieved in this way (see also Byerly & Hagiwara, 1982). Preliminary experiments indicate that this outward current involves an aselective cation channel (permeable to Cs) with Ca-dependent activation, since Ca-blockers that block the inward current, also completely abolish this outward current).

Whole cell clamp recordings were subsequently used to study the effect of 8-CPT-cAMP on the Ca-current. With Cs-saline in the pipette (in mM: 50CsCl, 10^{-8} $CaCl_2$, 10 Hepes, 7 CsOH, pH 7.6) and Ca/Cs-saline (standard saline with Cs replacing Na and K) containing 3x 10^{-5} M TTX in the bath, a Co-sensitive inward current remained that was increased up to twofold in amplitude by 8-CPT-cAMP. At potentials of >0 mV, however, the above described outward current obscured the Ca-current. Replacing Ca by 10 mM Ba increased the inward current, whereas the outward current was suppressed by replacing CsCl in the pipette by TEACl. Under these circumstances an almost completely isolated Ca-current was recorded. This current had the following characteristics (HP -90 or -70 mV) (Fig. 6): activation only ensued at command potentials of -10 mV and higher, the peak current occurring at 0 mV,

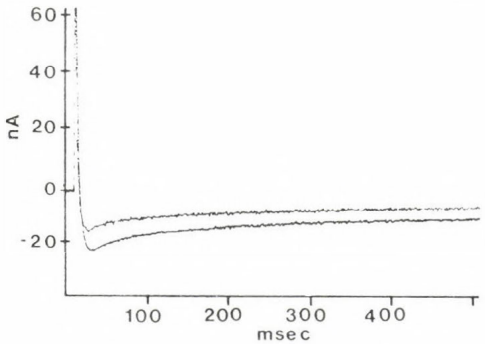

Fig. 5.Current responses of a Cs-loaded GHC to step depolarizations before and after application of 10^{-4} M 8-CPT-cAMP. 8-CPT-cAMP clearly increases the inward (Ca-)current. (Two electrode voltage clamp, HP -60 mV, command potential 0 mV; cells loaded with 60 mM CsCl, 9.5 $MgCl_2$, 40 sucrose, 10 Hepes; bath: standard saline with Cs replacing K and Ba replacing Ca, 3x 10^{-5} M TTX; pH 7.2.)

showing a very steep voltage dependence. Rapid inactivation was very limited, peak inward and late inward current differing by less than 20%. The peak inward current reversed at +55 mV, the late inward current at +50 mV, confirming that the current was almost completely pure. Ca-blockers, such as 10^{-3} M Ni, completely blocked the current. The characteristics of the Ca-current described here are in fair agreement with the results of Byerly & Hagiwara (1982), studying Ca-currents in unidentified neurones of *Lymnaea*. The lack of inactivation of the Ca-current suggests that, to a great extent, it is carried through L-type like Ca-channels (cf. Nowycky *et al.*, 1985; McCleskey *et al.*, 1986). This suggestion is born out by preliminary experiments, showing that the current is not inactivated when holding potentials of -50 or -40 mV are applied. Application of 8-CPT-cAMP caused an increase of this current, that was again completely blocked by 10^{-3} M Ni, and shifted the activation range by 10 mV in the negative direction (Fig. 7A). Similar experiments using standard saline in the bath confirmed these results (Fig. 7B). It is concluded from these experiments that cAMP enhances the Ca-current. It was also observed that the washout of the Ca-current, normally being 50% after one hour, was greatly diminished after application of 8-CPT-cAMP. With 8-CPT-cAMP in the bath the Ca-current could be recorded for over 2.5 hrs. The latter result is in line with observations on dorsal root ganglion cells (see Reuter, 1983).

The results reported here demonstrate that cAMP dependent regulation of the membrane excitability may profoundly affect the functioning of the GHCs. Since the effects of increasing the intracellular level of cAMP were observed both in current clamp and two electrode clamp recordings of GHCs in the central nervous system, as well as under whole cell clamp conditions, cAMP dependent modulation of ionic currents is likely to be a physiological mechanism in these cells. This is the more so, since it is known that these cells possess dopa-

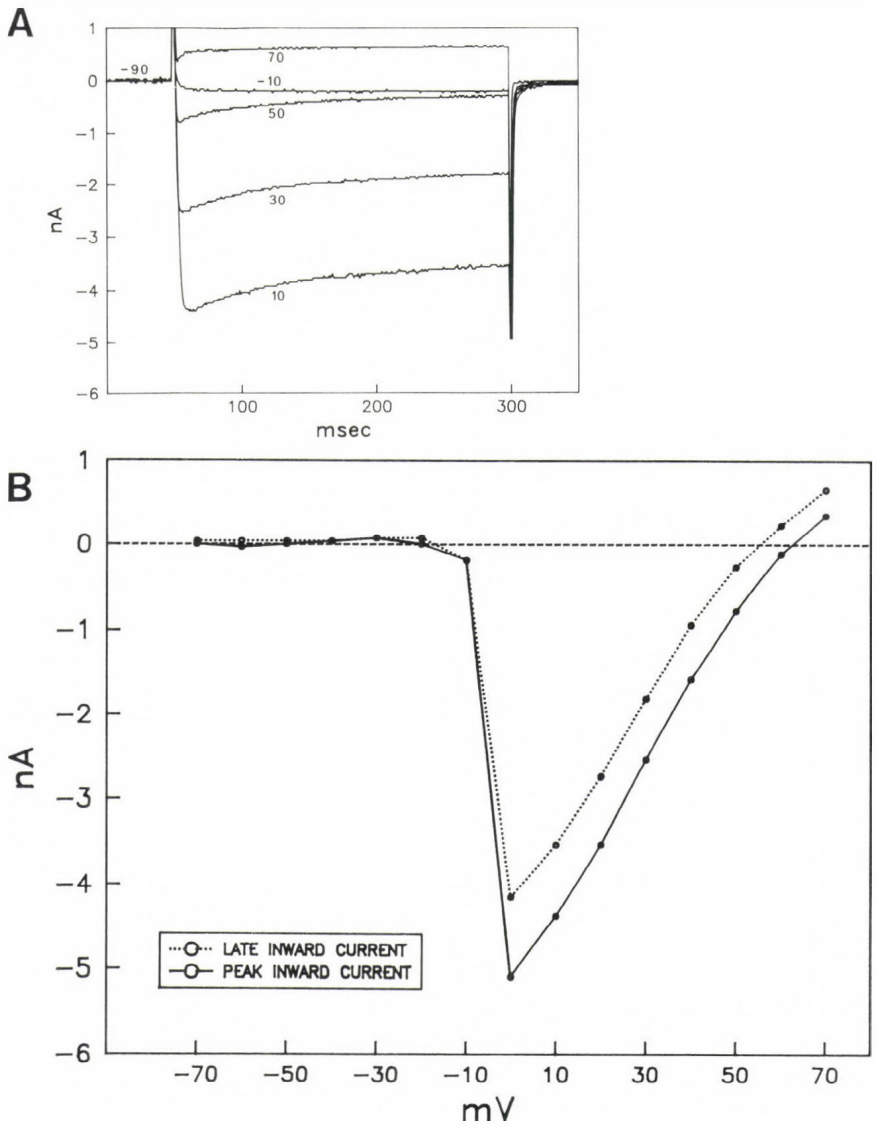

Fig. 6. A. Ca-currents under whole cell voltage clamp evoked by step depolarizations of -10 to +50 mV. (HP -90 mV.) B. IV-relationship of the peak and late Ca-current obtained by whole cell voltage clamp recordings. Same data as Fig. 6A (Pipette: TEA-saline; bath: Ba/TEA-saline, HP -90 mV.)

mine D-1 and D-2-like receptors, mediating responses to dopamine that are partly cAMP dependent (Stoof *et al.*, 1984).

The action of 8-CPT-cAMP on the peptidergic GHCs described here is similar to the effect of 8-bromo-cAMP on the Ca-current in cardiac cells (Cachelin *et al.*, 1983). In the latter pre-

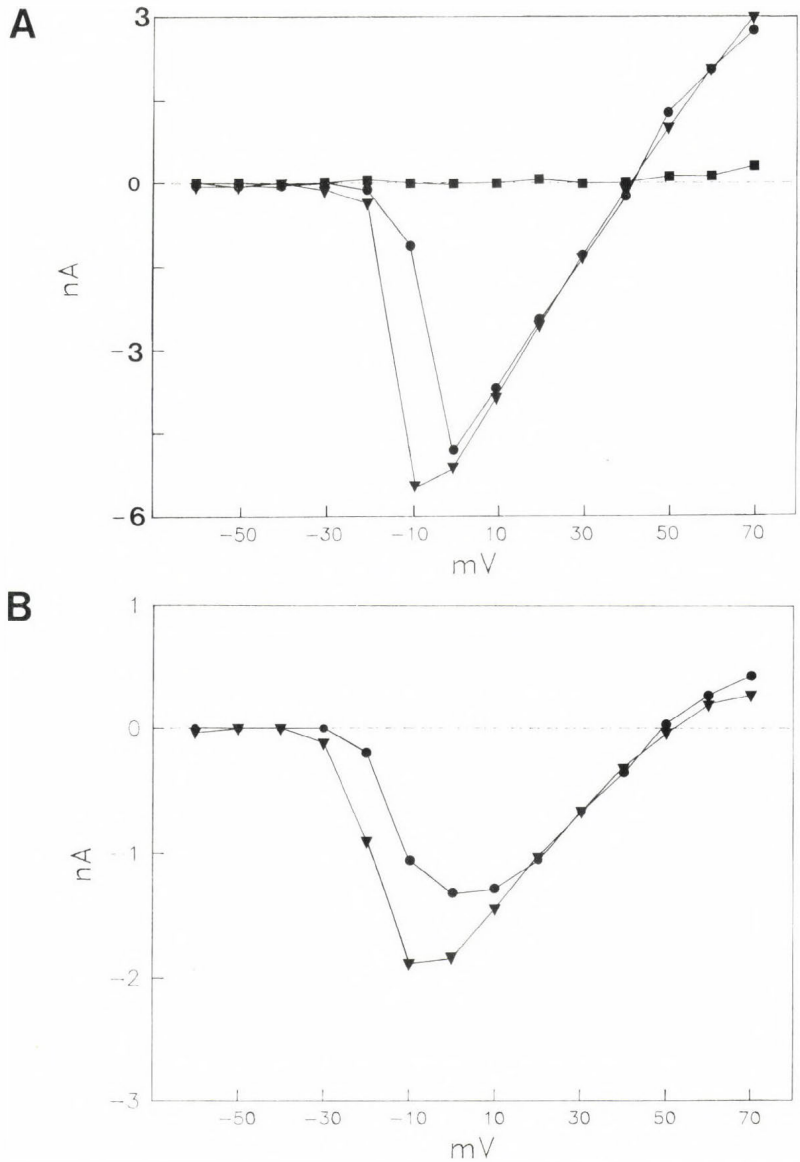

Fig. 7. A. IV-relationship of peak Ca-current before (●) and after application of 10^{-4} M 8-CPT-cAMP (▲). Addition of 10^{-3} M Ni completely blocks the Ca-current (■) (HP -70 mV; salines as in Fig. 6). B. IV-relationship of peak Ca-current, obtained with standard saline (containing 4 mM Ca and no Ba) in the bath, before (●) and after application of 10^{-4} M 8-CPT-cAMP (▼). (Otherwise like A.)

paration, cAMP dependent phosphorylation of L-type Ca-channels is believed to be induced by activation of β-adrenoceptor agonists (Osterrieder *et al.*, 1982; Reuter *et al.*, 1986),

whereas cGMP antagonizes the cAMP dependent increase in Ca-current (Hartzell & Fischmeister, 1986). In molluscan neurones a variety of modulatory actions on the Ca-current, employing protein kinase C (cf. DeRiemer *et al.*, 1985), cGMP-dependent protein kinase (cf. Paupardin-Tritsch et al., 1986) or cAMP mediated mechanisms (this study), have been demonstrated. It is well conceivable in GHCs that stronger potentiation of the Ca-current (possibly carried by different Ca-channels), through one of the other mechanisms may constitute a prerequisite for the occurrence of discharge-like activity.

The effect of increasing the intracellular level of cAMP can be described as a shift from a predominantly Na-dependent electrical behaviour to a predominantly Ca-dependent behaviour. In other words, the cells become Ca-oriented: control spikes hardly possess a Ca-component, whereas after cAMP treatment it appears that spiking is hardly affected by TTX. The differences in characteristics between the Na-current and the Ca-current satisfactorily account for the difference in excitability. The decrease in spiking threshold is explained by the combined activation of Na and Ca-channels after cAMP treatment, providing much stronger excitation of the cell. In this respect the shift of the activation range of the Ca-current towards lower membrane potentials is thought significant. The decrease in accommodation rate is explained by the slow and limited inactivation of the Ca-current, contrary to the rapidly inactivating Na-current.

Acknowledgements. The authors thank Mrs. T. Laan for her help in preparing the manuscript. KSK was supported by a C. & C. Huygens-grant from the Dutch Organization for the Advancement of Pure Research (ZWO).

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DISCUSSION

BENJAMIN, P.R.: Did you find the short afterdischarges in GHCs that we described some time ago?

KITS, K.S.: Yes, when the GHCs are kept in culture for 24 hours or more, they exhibit increased excitability and often short afterdischarges can be evoked.

BLANKENSHIP, J.E.: Did you record from GHCs in young and small animals and did you find increased excitability in that case?

KITS, K.S.: We have not done these experiments yet, but it would be certainly interesting to carry them out.

ELLIOTT, C.J.H.: You mentioned a drop in action potential threshold when you cultured the nervous system. Is this in the entire CNS?

KITS, K.S.: Yes.

ELLIOTT, C.J.H.: What brings about the change in threshold?

KITS, K.S.: We have not measured the cAMP levels in the cells.

TAUC, L.: Did you find similar modulation of the Ca-current by cAMP in other cells?

KITS, K.S.: Up till now we have studied the Growth Hormone Cells and the Caudo-Dorsal Cells that release the ovulation hormone. In the Caudo-Dorsal Cells increasing the level of intraceullular cAMP also enhances excitability. We have, however, not been able to establish an effect of cAMP on the Ca-current in these cells.

WINLOW, W.: 1. These are most exciting findings. The changes in cyclic AMP levels which modulate the spike activity by increasing the voltage-dependent Ca-current probably explains the gross alterations in the activity patterns of these cells which we believe to be a seasonal variation. Can you speculate as to which substance or substances might be responsible for modulating the cyclic AMP levels.

2. If dopamine is the modulator for changes in cyclic AMP levels, do you have any suggestions as to its likely source?

KITS, K.S.: 1. We have no conclusive evidence as to what transmitter or modulator actually evokes the enhancement of the Ca-current via increasing cAMP levels. We do know, however, that the GHCs have dopamine receptors, i.e. both a D-1-like and a D-2-like dopamine receptor. The D-2-like receptor is negatively coupled to an adenylate cyclase, whereas some indications have been obtained that the D-1-like receptor is positively coupled

to an adenylate cyclase, which would be analogous to the situation in vertebrate neurones. The effect of dopamine on the Ca-current is now under investigation.

2. No. We have demonstrated, however, that dopamine-positive immunoreactivity is closely associated with the axons of GHCs in the lip nerves.

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THE METABOLIC REGULATION OF MEMBRANE CHEMOSENSITIVITY

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Currently numerous experimental data are available on the metabolic regulation of excitable membrane functions. This regulation is realized either by the change in the number of functionally active protein molecules in the membrane or by the modulation of their activity.

In our earlier works it was shown that the electrogenic Na-pump is the main mechanism by which the metabolism regulates cell volume. This regulation has a physiological significance changing the membrane permeability, excitability (Ayrapetyan and Suleymanian 1979), chemosensitivity (Ayrapetyan and Arvanov 1979) and the number of pump units in the membrane (Ayrapetyan 1980). It turned out that protein molecules are in functionally active and inactive states depending on membrane packing: cell swelling leads to an increase while shrinkage to a decrease in the number of functionally active protein molecules in the membrane. Thus the electrogenic Na-pump, besides the regulation of intracellular ionic homeostasis, can also regulate metabolically the excitability, chemoreceptivity and enzymatic activity of the membrane changing the effective cell surface.

The second mechanism through which the Na,K-pump can regulate the functional activity of the cell membrane is the metabolic change in the cytoplasm and in the membrane as a result of changing both in intracellular ionic composition and in ATP/ADP+P ratio. Concerning the phosphorylation and dephosphorylation processes, the correlation between the activity of the Na-pump and the different intracellular messenger systems (cy-

clic nucleotides, kinase C and Ca ions) and the degree of membrane phosphorylation were studied.

The results of the investigation are presented below.

DEPENDENCE OF INTRACELLULAR cAMP AND cGMP LEVEL ON Na,K-PUMP ACTIVITY

The cyclic nucleotide content was determined in ganglia incubated both in normal physiological and in K-free or 10^{-4} ouabain containing solution.

As shown in Table 1, the inhibition of Na,K-pump induced by K-free or ouabain containing solutions increased the intracellular cAMP level by 164% and 55%, respectively. Addition of the ouabain to the K-free solution led to a 138% increase in intralular cAMP level as compared to the control. The different effectivities of both factors on the level of intracellular cAMP concentration could be explained either by their different effects on membrane potential (K-free saline hyperpolarizes and ouabain depolarizes the membrane) or by the fact that ouabain could immediately inactivate adenylate cyclase or activate phosphodiesterase. Still, more detailed investigations are needed to clarify the reason of different effectivities of the K-free saline and ouabain on intracellular cAMP level.

Table 1. Effect of Na,K-pump inhibition on the cAMP and cGMP intracellular levels

Conditions	cAMP level (pmole/mg protein)	Per cent of increase	cGMP level (pmole/mg protein)	Per cent of decrease
Normal physiol. sol.	7.5 ±0.82	-	3.80±0.42	-
K-free sol.	19.77±2.66	164	2.62±0.14	29
Ouabain 0.1 mM	11.61±0.89	55	2.71±0.25	30
K-free + ouabain	17.84±2.12	138	-	-

The cGMP level decreased by 30% compared to the control both in the K-free saline as well as in the presence of ouabain.

Thus Na-pump inactivation caused a decrease of cGMP and an increase of cAMP concentrations. The next series of experiments were devoted to studying the effects of ouabain and K-free saline on the degree of membrane phosphorylation.

DEPENDENCE OF THE DEGREE OF MEMBRANE PHOSPHORYLATION ON Na,K-PUMP ACTIVITY

Membrane phosphorylation was studied in membrane fractions incubated in (γ-^{32}P)-ATP containing medium, and in (^{32}P-H_3PO_4 containing medium.

In the first series of experiments when the extracted membranes were incubated with (γ-^{32}P)-ATP, the incorporation of ^{32}P into the membrane was decreased by 35% after 5 min in the presence of 10^{-4} M ouabain as compared to the basal phosphorylation level (Table 2a).

In the second series of experiments, when ganglia were incubated with (^{32}P)P_N, an increase of membrane phosphorylation by 38% was observed in the presence of K-free-ouabain containing saline (Table 2b).

Thus under in vivo conditions inactivation of the Na,K-pump increases membrane phosphorylation, while direct inactivation of membrane Na,K-ATP by ouabain decreases the degree of phosphorylation.

These data allow us to suggest that ouabain also has a direct inhibitory effect on the membrane phosphorylation processes, the molecular mechanism of which still awaits clarification.

The ouabain-induced inhibition of phosphorylation of the squid optical nerve membrane was observed by Matsumura (1977), while Raukch and Rososki (1984) observed an activating effect of ouabain on protein phosphorylation following detergent treatment. The data show that ouabain has a double effect on the degree of membrane phosphorylation. Pump inhibition leads to an increase in cAMP concentrations and membrane phosphoryl-

Table 2. Effect of Na-pump inhibition on the phosphorylation of membrane fractions

a) Membrane fractions incubated with (γ-^{32}P)-ATP

Conditions	^{32}P incorporation in membrane fraction (pmole of ^{32}P/mg prot.)	Per cent of inhibition
Basal phosphorylation level	$895.6\times10^{-6}\pm68.2\times10^{-6}$	-
Ouabain 10^{-4} M	$585.6\times10^{-6}\pm37.2\times10^{-6}$	35

b) Whole ganglia incubated with (^{32}P)-H_3PO_4

Conditions	^{32}P incorporation in membrane fraction (pmole of ^{32}P/mg prot.)	Per cent of activation
Normal physiol. solution	$254.8\times10^{-3}\pm20.5\times10^{-3}$	-
Ouabain 10^{-4} M	$350.0\times10^{-3}\pm10.2\times10^{-3}$	37.5

ation on the one hand, while ouabain itself decreases membrane phosphorylation, on the other hand.

The data presented above reveal that there is a close correlation between the Na,K-pump activity, the intracellular level of cyclic nucleotides and the degree of membrane phosphorylation. Thus it can be suggested that under normal conditions of the cell the variation in Na,K-pump activity is accompanied by a corresponding change in the degree of phosphorylation of membrane proteins. Presently it is known that phosphorylation of membrane proteins determines the intracellular concentration of another second messenger, the Ca ions. Ca ions enter into the neuron via the potential-dependent ion channels and via the Na-Ca exchange mechanism. It was established that both transport mechanisms are stimulated enhancing the intracellular cAMP concentration (Doroshenko et al. 1982, Bittar 1983).

COUPLING OF Na,K-PUMP AND Na-Ca EXCHANGE
IN SNAIL NEURONS

It is known that Na^+ efflux consists of two components: an ouabain-sensitive and an ouabain-insensitive one. The ouabain-sensitive component is a Na,K-pump while the ouabain-insensitive component is represented mainly by Na-Ca exchange mechanism. It has been evidenced that the significance of Na-Ca exchange mechanism prevails over the influx of Ca ions through ionic channels, and that plays a key role in regulations of the intracellular homeostasis of Ca ions (Mullins et al. 1981). Recently it has been shown that there is a close correlation between Na,K-pump activity and Na-Ca exchange mechanism (Sagian 1987). Inhibition of Na,K-pump activity increases while its stimulation decreases Na-Ca exchange. As shown by Bittar (1983), in barnacle muscle fibres the Na-Ca exchange is a cAMP-dependent process. The repetition of this work in our laboratory on snail neurons proved the above conclusion of Bittar. Factors which increased cAMP concentration in the cell had a stimulating effect on Na-Ca exchange, too. For instance, very low concentrations (10^{-11} - 10^{-8} M) of acetylcholine (ACh) and gamma-aminobutyric acid (GABA) increased the ouabain-insensitive Na-efflux in snail neurons (Dadalian et al. 1987).

Figure 1 demonstrates that inhibition of Na,K-pump by ouabain-containing K-free solution leads to an increase in the Ca uptake by 60%. This potentiating effect of Na-pump inhibition on Ca uptake was blocked at low temperatures. It was also demonstrated that factors such as GABA, lectin concanavalin A and atropine, which influence the chemosensitivity of the membrane (see below), have a modulatory effect on Ca uptake, too. These data allowed us to conclude that intracellular Ca ion concentration is actively regulated by the Na,K-pump.

It is well known that insignificant changes in intracellular Ca concentration can lead to dramatic changes of some enzymatic activities in the cell. In the abundance of Ca-dependent enzymatic reactions an important role is attributed to the phospholipase C which catalyses the hydrolases of phosphatidylinositol-(4,5)-biphosphate to inositol-(1,4,5)-triphosphate and

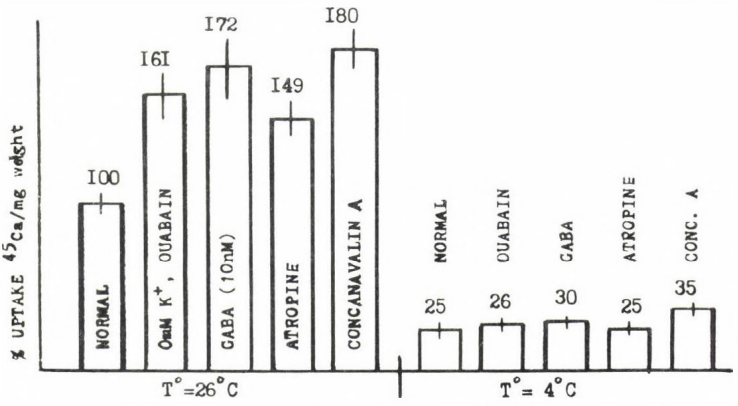

Fig. 1. Effects of ouabain, GABA, atropine and concanavalin A on $^{45}Ca^{2+}$ uptake processes

diacylglycerol. Both products have intracellular messenger functions (Berridge 1984). The first releases Ca from the intracellular pools, and the second activates protein kinase C, which in turn regulates numerous important processes of the cell, including its chemoreceptive properties (Oron et al. 1985, Vicentini et al. 1985, Adams and Brown 1986). A close correlation between cyclic nucleotide concentration and kinase C activity has also been demonstrated (Nishizuka 1983, Schwartz et al. 1986, Taylor and Merritt 1986).

It can be suggested, therefore, that the inhibition of Na,K-pump activity is quite sufficient to switch on the intracellular cAMP messenger system, which is followed by an increase in the degree of membrane phosphorylation, increase of Ca influx through potential-dependent ionic channels and Na-Ca exchange, the activation of phospholipase C and protein kinase C. The validity of this suggestion is demonstrated by studies of the correlation between the Na,K-pump activity and the chemosensitivity of the neuronal membrane.

Na,K-PUMP ACTIVITY AND THE AFFINITY OF MEMBRANE CHEMORECEPTORS

Recently we have shown (Arvanov and Ayrapetyan 1980) that cardiac glycoside ouabain, a specific inhibitor of Na,K-ATPase

activity, has a blocking effect on the ACh-induced responses of Helix neuronal membranes. As shown by Carpenter et al. (1976) in membrane fractions of Aplysia central nervous system and by us in intact neurons of Helix ganglia (Ayrapetyan et al. 1985), the blocking effect of ouabain on transmitter-induced membrane responses is a result of its blocking action on the corresponding membrane receptors. Two types of Helix neurons studied by intracellular dialysis are distinguishable by their ouabain sensitivity. In response to ACh two types of neurons were distinguished: A-type neurons, in which the ACh responses are almost completely blocked by ouabain and the response is Ca- and Na-dependent; and B-type responses which are not blocked by ouabain and are Na^+ and K^+ dependent.

Our data (Arvanov et al. 1984) on the depression of ACh responses both by ouabain and K-free solution allowed us to conclude that the effect of cuabain on membrane chemosensitivity is due to its inhibitory effect on Na,K-pump activity.

The Na-pump is a potent consumer of ATP and therefore it can be important in governing the ATP level in the membrane. There is literary evidence that membrane chemosensitivity depends on intracellular ATP concentration (Nistratova 1980, Andreev et al. 1986). The question arises if an increase in intracellular ATP concentration induced by inactivation of the Na-pump can modulate membrane ACh sensitivity.

As shown in Fig. 2, an increase in the internal ATP concentration leads to a decrease in both ACh- and GABA-induced membrane currents in the A-type neurons. The addition of ATP to the internal solution also causes a decrease in transmitter-induced responses but its inhibitory effect is rather weak compared to the ATP effect. An increase in internal AMP concentration had no visible effect on transmitter-induced currents. It should be mentioned that in the case of B-type neurons (where ACh responses are not depressed by ouabain) the inhibitory effect of ouabain on ACh-induced currents was absent. It is interesting to note that adenylylimidodiphosphate, a non-hydrolysed analogue of ATP, had an ATP-like inhibitory effect on A-type ACh responses. These data suggest that free ATP may play

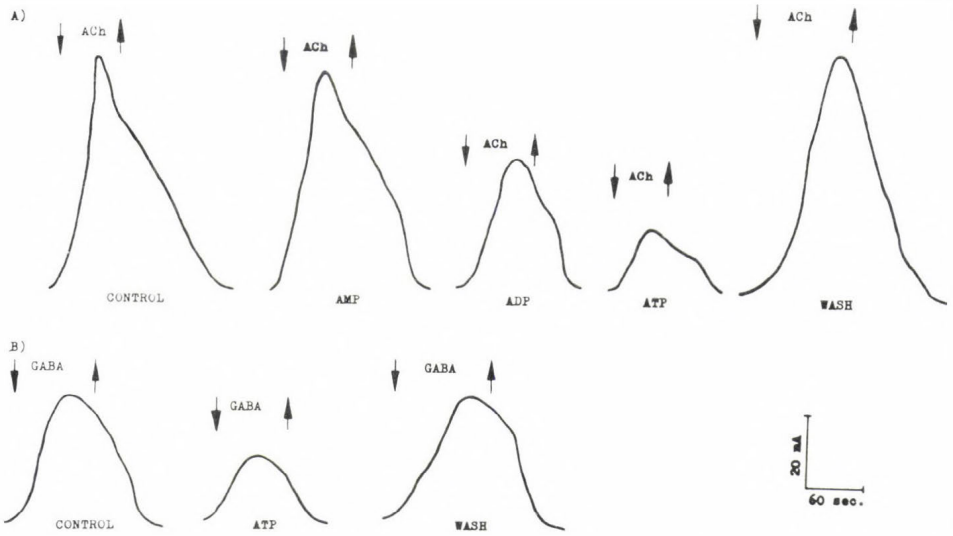

Fig. 2. Effects of intracellularly applied adenosinephosphates on ACh (A)- and GABA (B)-induced membrane currents

the role as a modulator of the chemoreceptive properties of the neuronal membrane.

As shown in Fig. 3, the depressing effect of internal ATP on the ACh responses of the A-type neurons is absent in the presence of internal dinitrophenol (DNP). In the same Figure it can be seen that in the presence of internal ATP and at low temperatures the blocking effect of ouabain on ACh responses disappeared.

To find out whether the ouabain-induced enhancement of the internal concentration of Ca ions (see Fig. 1) may participate in the realization of ouabain effect on membrane chemosensitivity, the influence of internally added EGTA on A-type ACh responses was studied. As shown in Fig. 4 the inhibitory effect of ouabain on ACh responses disappeared after the coupling of EGTA with internal Ca. In addition, it must be noted that a low concentration of GABA which increases cAMP level (Dadalian et al. 1987) also increases the Ca inward current through dialysed neuron membrane (Fig. 4A) and induces the same blocking effect on ACh responses as does ouabain (Fig. 4B). Figure 4B also shows that the inhibitory effect of both 10 nM GABA and

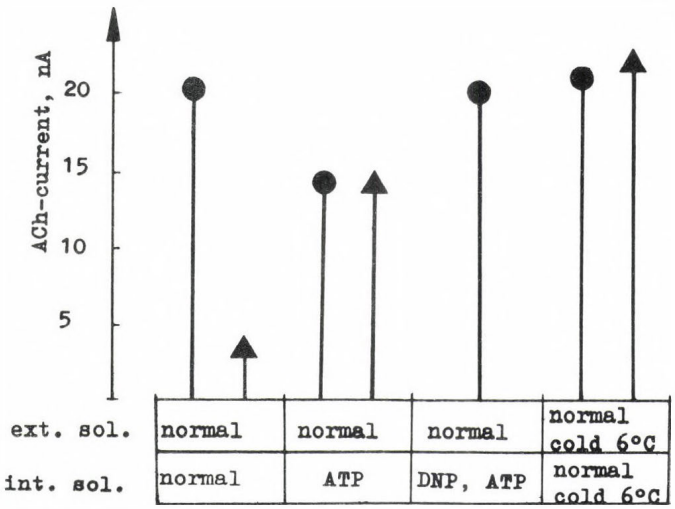

Fig. 3. Interaction of ouabain and intracellular ATP on ACh-induced currents of A-type neurons. ●, ACh current in the absence of ouabain; ▲, in the presence of ouabain

0.1 mM ouabain on ACh A-type responses disappeared in EGTA-containing internal solution.

As shown in Fig. 5, ouabain and K-free solution leads to a significant inhibition of ^{3}H-α-BT binding to the membrane receptors. Preincubation of the ganglion in a cold K-free solution leads to an insignificant inhibition of the binding. It must be noted that the inhibitory action of warm K-free solution on ^{3}H-α-BT binding increases with the time of preincubation, while in the case of cold K-free solution it is independent of the time of preincubation.

These data revealed that ouabain- and K-free-induced inhibition of α-BT binding is mediated by the energy-dependent metabolic processes which regulate the receptor's affinity for neurotransmitters. Such metabolic mechanism could be the intracellular ATP-modulated membrane receptor phosphorylation.

In order to check this suggestion the binding of α-BT to membrane receptors at different conditions was studied.

It is well known that in snail neuronal membrane two types of cholinoreceptors are present: nicotinergic (N) and muscarinergic (M) (Walker and Kerkut 1977). In order to distinguish

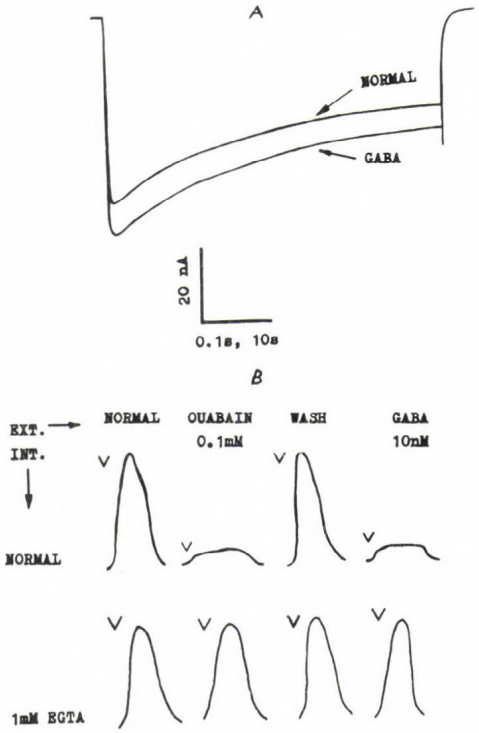

Fig. 4. Effect of 10 nM GABA on Ca inward current (A) and on ACh-induced current (B)

the pathways of the affinity regulatory mechanism of the N and M receptors, the binding of α-BT in both atropine- and d-tubocurarine (d-TC)-containing medium was studied. As shown in Table 3, the ouabain-induced pump inactivation leads to a depression of α-BT binding with M cholinoreceptors but has no effect on the affinity of N cholinoreceptors to α-BT. The activation of kinase C by phorbol ester causes a decrease of N cholinoreceptor affinity but has no effect on M cholinoreceptor affinity to α-BT. Applying both ouabain and phorbol ester the inhibitory effects on α-BT binding cumulate. The data on α-BT binding processes depending on the interaction of kinase A, kinase C and calmodulin systems show a close correlation of these systems in the regulation of α-BT binding with the membrane. In these experiments kinase A activity was inhibited by

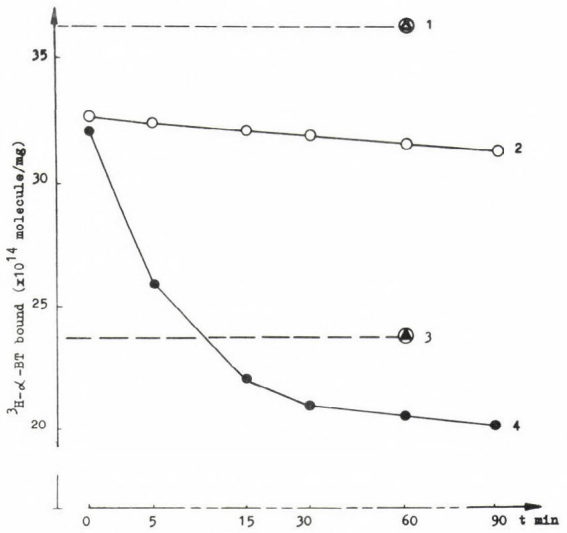

Fig. 5. Effect of ouabain and K-free solution on ^{3}H-α-BT binding. Before incubation with the ligand the ganglia were incubated in (1) normal, (2) K-free cold (6°C), (3) ouabain-containing and (4) K-free warm (26°C) solutions

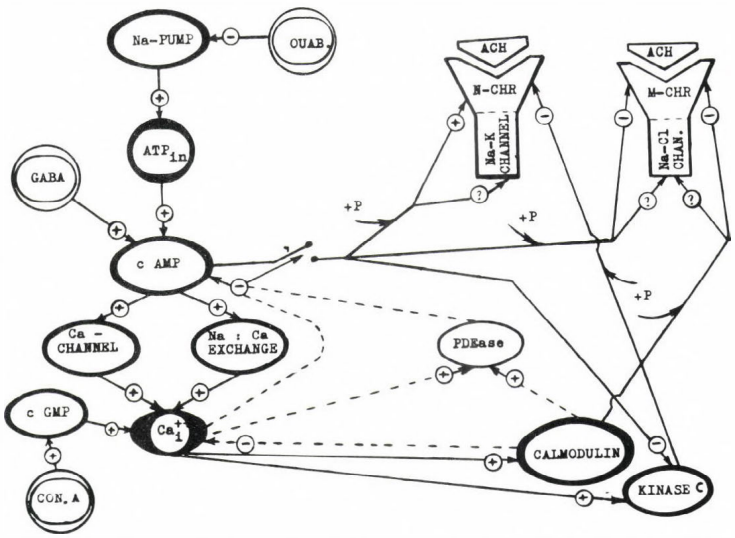

Fig. 6. A possible mechanism of Na,K-pump-induced regulation of membrane ACh chemosensitivity via cAMP, Ca uptake and kinase C system feedback control

Table 3. Dependence of ^{3}H-α-BT binding to the membrane on Na-pump, kinase A, kinase C and Ca-calmodulin activity

Conditions	Normal	Per cent inhib.	Atropine	Per cent inhib.	d-TC	Per cent inhib.
Control	204±21	-	207±22	-	123±11	- (40)
Ouabain (OUAB)	141±11	31	203±20	0	83±8	32
Phorbol ester (PHE)	153±12	25	149±12	28	120±10	0
PHE + OUAB	101± 8	50				
Trifluoperazine (TFP)	112± 9	45				
Theophylline	106± 8	48				
NaF	124± 9	39				
Tolbutamine	201±20	-				
Tolbutamide + TFP	177±15	11				

Table 4. Effects of manipulations of membrane phosphorylation state on the inhibitory action of ouabain on ^{3}H-α-BT binding

Conditions under which ouabain was added	Per cent ouabain-induced inhibition (-) and activation (+)
Control	-31
K-free solution	0
Cold (5°C)	0
Theophylline	0
NaF	-14
Tolbutamide	+36
Trifluoperazine	0
Atropine	0
d-TC	-32
Phorbol ester	-34

tolbutamide (Kanamoni et al. 1976) and kinase C and Ca-calmodulin activities were inhibited by trifluoperazine (Kaczmarek 1986).

As shown in Table 4, the effect of ouabain on α-BT binding processes can be modulated depending on the interaction of different kinase systems of the cell. On the basis of the obtained data a hypothetical scheme was suggested which explains the mechanism of interaction between Na,K pump activity and messenger-induced changes in membrane chemosensitivity (Fig. 6).

The data presented suggest that there is a close correlation between the Na,K-pump activity and the chemosensitivity of neuronal membrane, which is realized through the system of second messengers.

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DISCUSSION

ALKON, D.L.: Is it not possible that one or more of the kinase-regulated pathways could interact in the control of neuronal membrane chemosensitivity? Would it be possible to use a radioactive tag to investigate whether or not phosphorylation occurs on the chemoreceptors themselves or on molecules which regulate these receptors?

ARVANOV, V.L.: As it is revealed by our data the control of neuronal membrane chemosensitivity by the Na-pump is realized by its effect on the second messenger systems: inhibition of the Na-pump leads to the increase of internal cAMP concentration, internal Ca concentration and, finally, to the decrease of muscarinic receptor affinity to ACh, which is determined by the cAMP-dependent phosphorylation of membrane proteins. As to nicotinic receptors, our data on the inhibitory effect of phorbol esters on ^{3}H-α-bungarotoxin binding point to the participation of kinase C in the control of nicotinic receptor affinity. The data on the effects of tolbutamide, trifluoperazine, phorbol esters and other effectors of the kinase systems suggest that at least kinase A, C and Ca-calmodulin regulated pathways could interact in controlling membrane chemosensitivity. But interaction between these phosphorylation systems in Helix neurons is not yet clear and now we are studying this question in our laboratory.

POSTERS

Symposia Biologica Hungarica 36 (1988)

CONCENTRATION CLAMP ANALYSIS OF THE GLUTAMATE-INDUCED CHLORIDE CURRENT IN ISOLATED APLYSIA NEURONS

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L-Glutamic acid (Glu) is an excitatory neurotransmitter at crustacean neuromuscular junction and in mammalian central nervous system. In Aplysia nervous system, Glu is also an inhibitory transmitter, inducing a chloride current (Kehoe, 1976). Bath application and iontophoretic application have been used to characterize the Glu responses in various species of animals. Kinetic studies with perturbation or fluctuation analysis are indispensable to elucidate the mode of interaction between the neurotransmitter and the receptor-channel complex. In the present experiments, we studied the kinetics of the Glu-induced chloride current in Aplysia neurons using the 'concentration clamp' technique.

Circumesophageal and abdominal ganglia of Aplysia kurodai were dissected and single neurons were isolated mechanically after digesting the surrounding capsules in artificial sea water containing dispase (10,000 protease unit/5 ml; Godo Shusei Co., Ltd., Japan) for 60-80 min at 37 °C. The artificial sea water had the following composition (mM): NaCl 450, KCl 10, $CaCl_2$ 10, $MgCl_2$ 55 and Tris 10 with pH adjusted to 7.8 with Tris base and HEPES. A suction pipette technique was used for voltage clamp and internal perfusion of the neurons. Glu was applied using the 'concentration clamp' technique, which enabled a rapid exchange of the external solution within a few msec (Akaike et al., 1986). It was, therefore, possible to analyze the activation and desensitization phases, and to obtain a precise dose-response curve because the I_{Cl} reached a peak before desensitization took place. To isolate the chloride current from sodium and potassium currents, Na and K ions in both external and internal solutions were replaced with Tris and Cs ions, respectively. The external solution had the following composition (mM): Tris-Cl 340, Tris-base 100,

CsCl 10, $CaCl_2$ 10 and $MgCl_2$ 55 with pH adjusted to 7.8 with HEPES. The internal solution: Tris-base 300, aspartic acid 270, CsCl 300 and EGTA 5 with pH adjusted to 7.2 with Tris-base and HEPES. The majority of cells used had a diameter of about 50μm and all experiments were carried out at room temperature (about 20 °C). Curve fittings and noise analysis were performed using a microcomputer.

Figure 1A shows that Glu (0.03 to 3 mM) induced a transient I_{Cl} which reached a peak before desensitization occurred, providing the precise

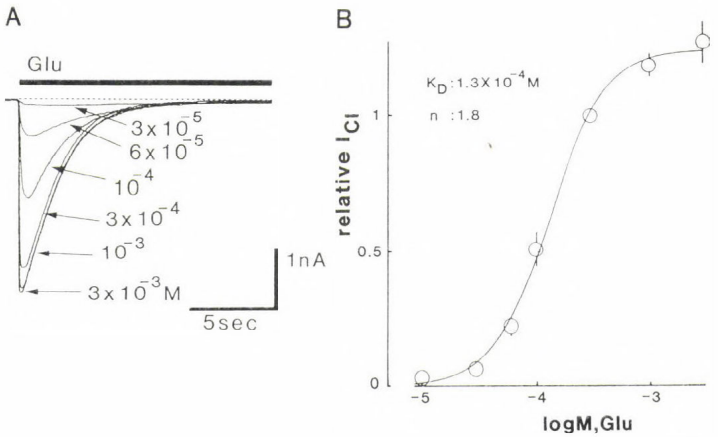

Fig. 1

A: Various concentrations of Glu induced rapidly activating and desensitizing I_{Cl} in a neuron voltage clamped at -30 mV.

B: The peak I_{Cl} was normalized to that evoked by 3 x 10^{-4}M Glu and plotted against the concentration. A least square fitting gave a dissociation constant (K_d) of 1.3 x 10^{-4}M and a Hill coefficient (n) of 1.8. The continuous line was drawn according to the following equation using those values and I_{max}= 1.3.

$$I = I_{max} \cdot \frac{C^n}{C^n + K_d^n}$$

where I is the observed peak I_{Cl}, I_{max} the maximum I_{Cl} and C the concentration of Glu. Each point is the mean of 5 to 7 experiments and bars indicate one standard error of mean when larger than the symbol.

amount of the activated channel. The I_{Cl} declined to null during a continuous application of Glu, indicating complete desensitization. Figure 1B illustrates the dose-response curve for the Glu-I_{Cl}. A least square fitting gave a dissociation constant of 1.3×10^{-4} M and Hill coefficient of 1.8. The I-V relationship was linear and the reversal potential for the I_{Cl} was -7.1 mV, which is close to the calculated reversal potential for Cl ions. This finding indicates a good internal perfusion and purity of the chloride current. The activation proceeded single exponentially, the time constant of which decreased with increasing concentrations of Glu (about 150 to 10 msec) but was not dependent on the membrane potential. The desensitization process was double exponential with a small second component but in most cases the process was fitted with a single exponential. The time constant of desensitization was concentration dependent (about 20 to 1 sec) but was not voltage dependent. The I_{Cl} recovered from desensitization double exponentially with time constants of 9.5 and 85 sec, which were not affected by changing the concentration of Glu. These results indicate two states of desensitization but it is not clear whether the two states are in parallel or in a series.

Conditioning pre-application of a lower concentration of Glu (50 μM) induced a desensitizing I_{Cl}. After a period of the pre-application, 1 mM Glu evoked a smaller I_{Cl} compared with the control without the pre-application. The smaller I_{Cl} represents the amount of the receptor-channel complex remaining activatable during the progression of desensitization. The remaining ratio decreased with the same time course as that of desensitization of the conditioning I_{Cl}. This finding suggests that a single component of the receptor-chloride channel complex was activated by Glu. Ensemble noise analysis during the activation of the I_{Cl} gave a single channel conductance of about 50 ps (n=4). Noise analysis was also performed on the desensitization process. The variance/I versus I (the average current) plot showed as if the number of available receptor-channel complex decreased with progression of desensitization.

In one cell out of eight which had Glu-I_{Cl}, quisqualate and kainate induced a small, non-desensitizing I_{Cl}, which did not cross-desensitize with the Glu-I_{Cl}. Aspartate did not induce I_{Cl} in 10 neurons which had Glu-I_{Cl}. Glutamic acid diethylester (GDEE) and jorospider toxin (JSTX) did not affect the Glu-I_{Cl} (Fig. 2). These findings indicate that the Glu

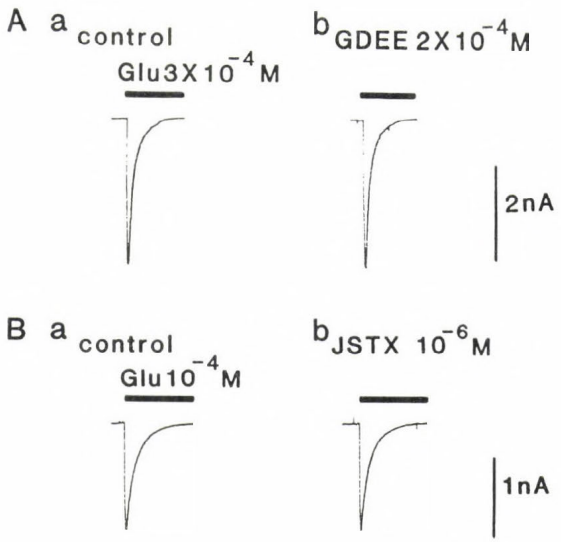

Fig. 2
GDEE (A) and JSTX (B) did not depress the Glu-induced I_{Cl}.

receptor-Cl channel complex in Aplysia neurons are different in nature from those in mammalian central nervous system.

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SEROTONERGIC INNERVATION OF THE PEDAL EPIDERMIS OF LYMNAEA

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Serotonin was located in frozen sections of the foot of Lymnaea stagnalis (L.) using the S.P.G. (glyoxylic acid) technique (de la Torre and Surgeon, 1976) and immunofluorescence through the biotin-avidin method (Hsu and Raine, 1981). Serotonergic axons were found to ramify through the foot and repeatedly bifurcate until they formed very fine tracts. These tracts extended to the base of the epidermis. Along the base were many bright varicosities (fig.1), often joined together by very fine tracts. The varicosities were very common under areas of ciliated epidermis but uncommon or absent under other areas of epidermis. These varicosities are presumed to be axon terminals which contain serotonin. Serotonergic cell bodies were only rarely seen outside of the brain and none were found associated with the epidermis. Both of the techniques used to demonstrate serotonin gave similar results, though more detail was visible using the immunofluorescence technique. Only non-specific, background fluorescence was seen in control sections.

Transmission electron microscopy of thin sections of foot demonstrated axon terminals in intimate contact with the base of ciliated cells (fig. 2). These terminals contained large, dense-cored vesicles as well as some smaller, more homogeneous ones. Similar terminals were also found on mucus-producing, goblet cells. What may have been serotonergic innervation of mucus cells was seen on frozen sections using immunocytochemistry. HPLC analysis of homogenized foot samples confirmed the presence of serotonin.

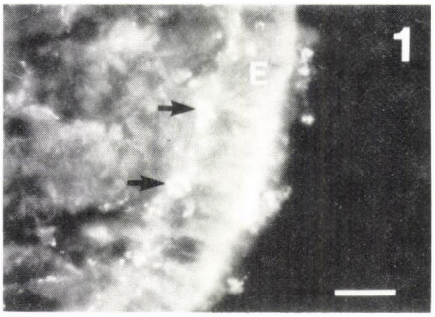

Fig. 1
Serotonergic immunofluorescence under the ciliated epidermis. E. epidermis, arrows point to serotonergic varicosities. Scale bar = 8.5 μm.

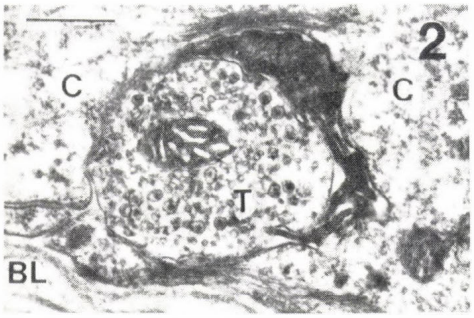

Fig. 2
Axon terminal in base of a ciliated cell. Note the dense-cored and lucent vesicles in the terminal. BL basal lamina, C ciliated cell, T axon terminal. Scale bar = 0.5 μm.

These results suggest that the ciliated cells of the epidermis are under neuronal control and that this control is at least in part serotonergic. Release of some forms of mucus may also be under neuronal control and both ciliary activity and mucus release may be co-ordinated by the same serotonergic neurons.

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DEMONSTRATION AND QUANTIFICATION OF SULFUR CONTAINING NEUROPEPTIDES IN THE CEREBRAL GANGLIA OF MELANOIDES TUBERCULATA (O.F. MÜLLER 1774, PROSOBRANCHIA, MESOGASTROPODA)

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In prosobranchs, compared with the two other subclasses of gastropods our knowledge of common neurosecretory phenomena is limited. This may be due to technical difficulties in staining neuropeptides by the classical methods and to the scattered occurrence of weak-coloured neurons (e.g. Nolte et al. 1965, Andrews 1968). However, results of organ culture experiments indicate the occurrence of a neuroendocrine factor for the sex reversal in Crepidula fornicata (e.g. Feral et al. 1987). Melanoides tuberculata is an ovoviviparous, subtropical freshwater snail with special strategies in parthenogenetic reproduction. A brood-pouch outside of the genital tract contains all developmental stages from eggs to juveniles (frequency of birth: 2/d) and is lined in part by epithelial nurse cells. Neuroendocrine control of reproduction may be postulated in such a complex system. Therefore, we tried a new approach to demonstrate peptidergic products within the nervous system of this species.

Histochemical demonstration

For this purpose the bromobimane technique (see Danielsohn, Nolte 1987) was applied, which is specific for sulfur-containing peptides. Using dibromobimane (DB) as the fluorescent labeling agent highly fluorescent reaction products were only formed within some neurons but not in glial cells of the cerebral ganglia. Fluorophores are not formed with any substance in the cytosomes. For the first time two distinct groups of perikarya of peptidergic neurons could be demon-

strated in each cerebral ganglion of this prosobranch snail. The groups are localized bilateral-symmetrically in a lateral and median position (Fig. 1). Besides, a few DB-positive neurons and axon swellings occur scattered within the cerebral ganglia.

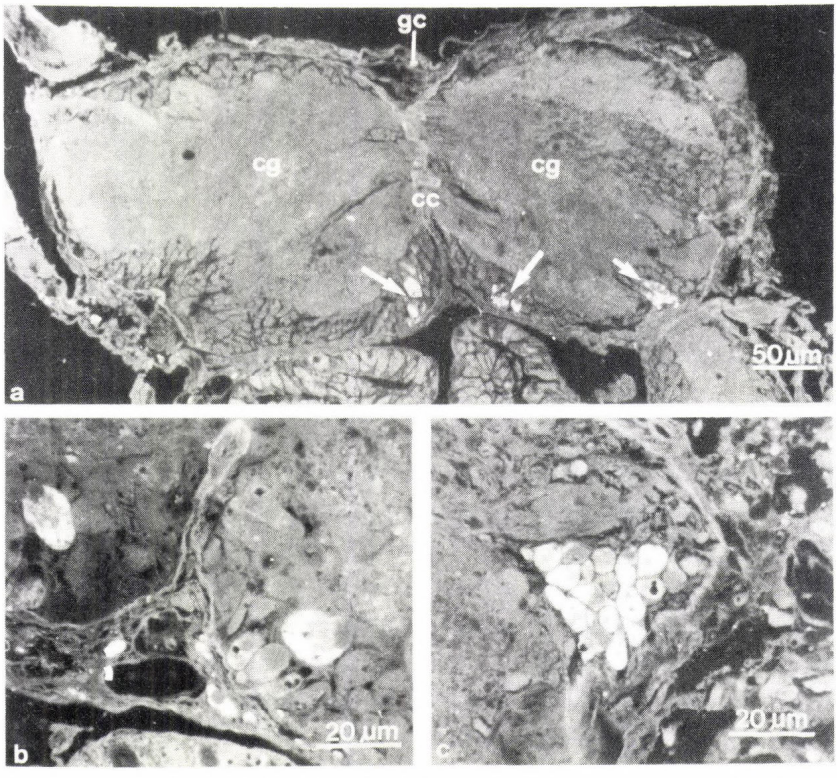

Fig. 1. Demonstration of sulfur-containing neuropeptides within semithin sections of the cerebral ganglia of Melanoides tuberculata labeled with dibromobimane (DB).
Excitation wavelength 380 nm, barrier filter 480 nm
a: cerebral ganglia (cg) with groups of neurons in a lateral (◄) and median (◄—) position; cc: cerebral commissure, gc: granular cell; exposure: 2 min
b and c: higher magnification of neurons of a median (b) and lateral group (c); exposure 30 s
Film: Agfa pan 400

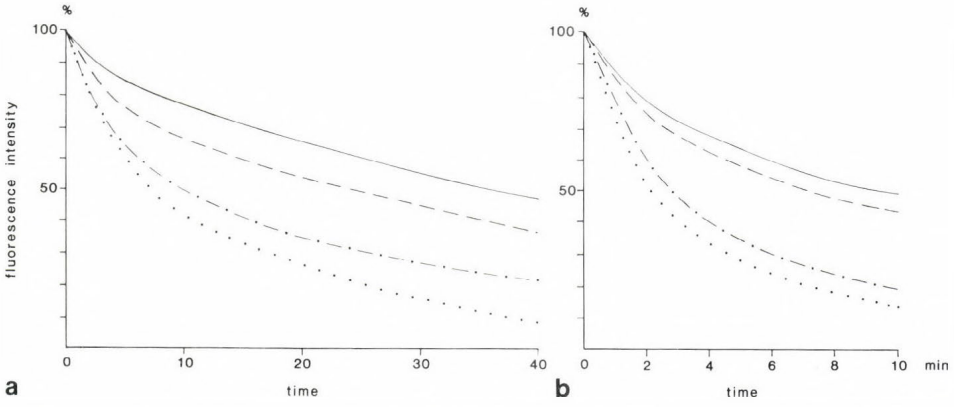

Fig. 2. Reduction of fluorescence intensity (fading) of DB-labeled neuropeptides dependent on different cover media (—— glycerol, – – – Citifluor, —•—•— UV-inert, ····· Immunomount). All data were calculated from three separate measurements. Excitation wavelength 380 nm, barrier filter 480 nm
a: using an objective Neofluar 16/0.40 (Zeiss)
b: using an objective Neofluar 40/0.75 (Zeiss)

Quantification

Using the sensitive bromobimane technique a quantification of the labeled sulfur-containing neuropeptides is possible by microspectrophotometric measurements. The contents of peptides can be analyzed by measuring the fluorescence intensity. For comparative measurements of these contents in special nerve cells or ganglia of specimens under experimental conditions the reduction of fluorescence intensity (fading) has to be considered. It is well known that fading is dependent on different parameters (e.g. fixatives, thickness of sections, duration of labeling, magnification and aperture of objectives, cover medium). To reduce the fading rate the influence of different cover media was tested: glycerol (Merck), Citifluor (Plano), UV-inert (Serva), Immunomount (Shandon). The fading rate dependent on different objectives was already demonstrated (Danielsohn, Nolte 1987).

The fluorescence intensity and its reduction were measured in 4 alternate semithin sections of the lateral group of neurons in the cerebral ganglia (Fig. 1c). Independent of the used objective the lowest reduction of fluorescence intensity was measured in glycerol, the highest one in Immunomount covered sections (Fig. 2). Generally, DB-labeled products fade slowly compared to other well-known fluorescent labeling reagents (e.g. fluorescein, rhodamine isothiocyanate fluorescamine). For comparative measurements parallel series of alternate sections are necessary, one series for the qualitative, the other for the quantitative investigations. Therefore, these four cover media could be applied for quantification, but all data have to be obtained in the first minute of UV-irradiation. We prefer UV-inert covered slides, because their storage and manipulation are much easier done compared to the other media.

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THE PHYSIOLOGY AND PHARMACOLOGY OF THE FEEDING INTERNEURONS OF THE POND SNAIL LYMNAEA STAGNALIS

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INTRODUCTION

The pond snail, Lymnaea stagnalis feeds by rhythmic protraction and retraction of its radula. The retraction phase is divided up into two phases, rasping and swallowing. Analysis of the rhythmic activity of the buccal neurons shows three phases of synaptic input which correspond with the three behavioural activities (Rose & Benjamin, 1981a). The synaptic input is produced by premotor interneurons in the buccal ganglia called N1, N2 and N3 neurons. Each type of premotor interneuron is active during only one phase of the rhythm, with the N1 neurons active during protraction, and the N2 and N3 interneurons in retraction. These interneurons have been characterised physiologically and anatomically (Rose & Benjamin, 1981b; Elliott & Benjamin, 1985). Their interactions are responsible for the rhythmic sequence of feeding activity.

THE PHYSIOLOGY OF THE PREMOTOR INTERNEURONS

1) Premotor interneurons are multi-action neurons. Each pattern generating interneuron produces synaptic potentials on the motoneurons as well as the other pattern generating interneurons. Spikes in a N1 interneuron are followed by PSPS in motoneurons (e.g. 5 and 1 cells, Fig. 1), even in a saline with elevated Ca and Mg ions. Successive PSPS always have nearly the same latency - there are no sudden jumps or alternation of short and long latencies. However, the latency depends on the somatic membrane potential between spikes and decreases (5ms - 1ms) as the cells depolarise. The short latency and persistence in 'high divalent' saline suggest that the PSPS are monosynaptic, although contributions from the other (electrically coupled) N1 cells (Rose & Benjamin, 1981b) cannot be ruled out.

The connections between the interneurons and motoneurons are summarised in Table 1. Note that some neurons (e.g. 3 cell, 7 cell) have antagonistic inputs from the N1 and N2 interneurons, but that where the cell receives

input from N2 and N3 interneurons both produce the same effect. This may be because the N1 neurons are used in protraction, while both the N2 and N3 cells take part in retraction.

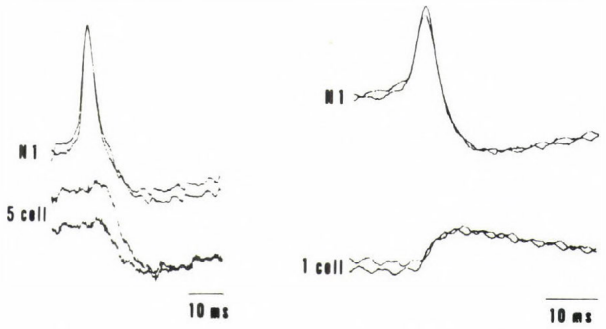


Fig. 1. Synaptic potentials produced by an N1 interneuron.

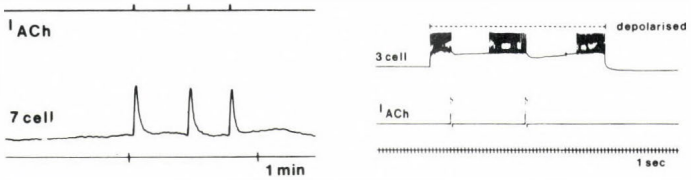
THE PHARMACOLOGY OF THE PREMOTOR INTERNEURONS

1) Cholinergic potentials. Even in a low calcium-high magnesium saline which blocks synaptic transmission in the buccal ganglia, each of the feeding motoneurons responds to acetylcholine (ACh) (Table 1). Application of ACh from a micropipette depolarises the 7 cell (Fig. 2a). In another preparation (Fig. 2b), depolarising current is used to activate the 3 cell and then ACh ejected iontophoretically. This hyperpolarises the cell and inhibits action potentials. The cholinergic potential had the same sign whether the ACh was applied from a micropipette or in the bath.

Table 1. Responses of the SO interneuron and of the feeding motoneurons

Cell type	SO	1	2	3	4	5	7	10
Response to ACh	e	e	e	i	i	i	e	e
Response to N1	e	e	e	i	i	i	e	e
Response to N2	i	-	e	e	i	i	i	e
Response to N3	i	-	-	e	i	i	i	-

Table 1 shows that the response of the motoneurons to ACh was always the same as to N1 stimulation. The 1 and 2 cells both have an excitatory response and are depolarised by each of the cholinomimetics carbachol, TMA and PTMA. The same agonists inhibited the 4 cluster cells, but the 3 cell was unaffected by PTMA. The 3 cell, unlike the 1, 2 or some 4 cluster cells, is inhibited by arecoline.



(a) (b)

Fig. 2. Responses to acetylcholine iontophoresis. (a) Intracellular recording from a 7 cell, depolarised by three ejections of ACh. (b) Intracellular recording from a 3 cell. For much of the record, the cell was depolarised by current injection (dotted line). Two current pulses were used to eject ACh, which hyperpolarised the 3 cell and inhibited it.

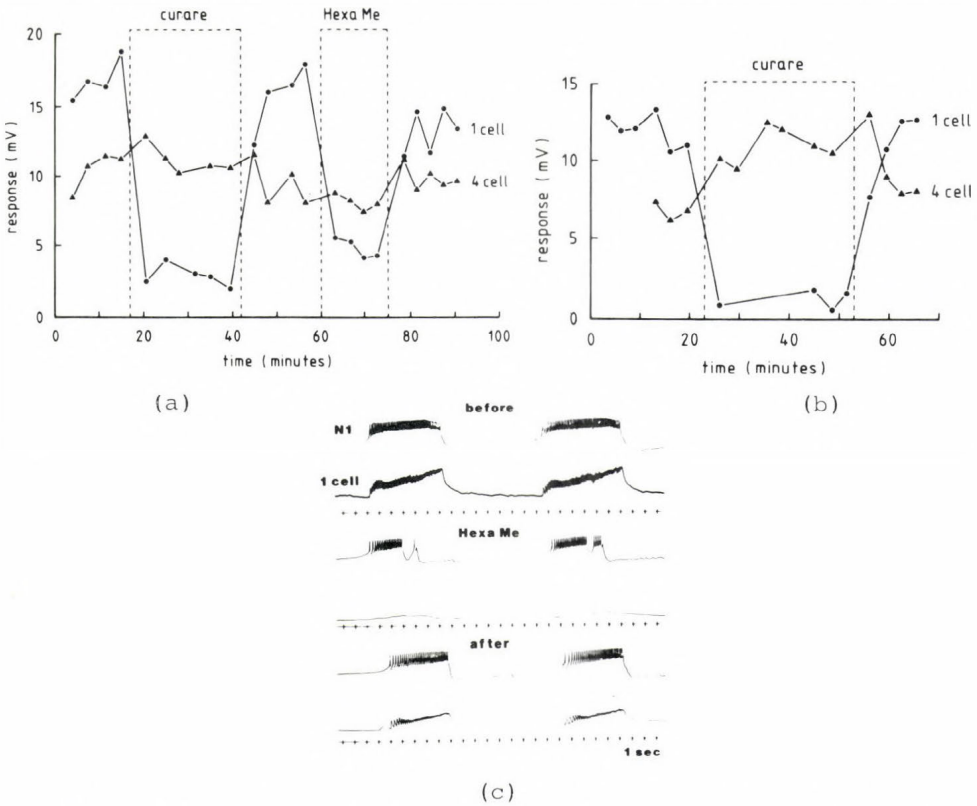


Fig. 3. The response to cholinergic antagonists. (a) ACh was applied in the bath in repeated pulses with the antagonists hexamethonium or curare, during intracellular recordings from the 1 cell and a 4 cluster cell. The latter was depolarised by 15mV. (b) As 'a', but the 4 cluster cell hyperpolarised. (c) Simultaneous intracellular recordings from an N1 interneuron and the follower 1 cell. The 1 cell EPSP is blocked by hexamethonium.

2) Cholinergic antagonists. During simultaneous intracellular recordings from identified motoneurons, ACh and antagonists were perfused through the bath.

Hexamethonium reduces the excitatory response in the 1 cell, but not the inhibitory response in the 3 or 4 cell (Fig. 3a). Curare has similar effects, except when the 4 cell is hyperpolarised. Then curare increases the response to ACh (Fig. 3b). The best explanation for this is as follows: the 4 cell has two inhibitory receptors and curare selectively blocks the part of the inhibitory response which has a reversal potential around -60mV. A third antagonist, PTMA, attenuates the ACh response of 1, 3 and 4 cells.

The antagonists hexamethonium and curare attenuate the Lymnaea cholinergic potentials. These same antagonists were next perfused while recording from a N1 interneuron and a follower motoneuron in a saline rich in Ca and Mg ions, which attenuates polysynaptic pathways.

Figure 3c shows recordings from an N1 interneuron with 1:1 EPSPS following in the 1 cell (top traces). Perfusing the cholinergic antagonist hexamethonium abolishes the EPSPS seen in a 1 cell (middle traces), but within 5 minutes of the washout, the EPSPS return. When applied in similar fashion, curare also blocks the 1 cell EPSP.

The N1 interneurons also make inhibitory synapses onto 5 cells. This synapse is also reversibly blocked by the perfusion of curare. This shows that the inhibitory as well as the excitatory effects of the N1 cells are likely to be cholinergic.

CONCLUSION

The evidence presented here shows that the N1 cells appear to make monosynaptic contact with feeding motoneurons. Those excited by the N1 interneurons are also excited by ACh and cholinomimetics. The motoneurons inhibited by the N1 neurons are also inhibited by ACh and some of the agonists. Two of the antagonists which block the cholinergic potentials block N1-induced synaptic potentials too. We conclude from this that the N1 neurons probably release ACh as their transmitter. It remains to demonstrate that the N1 cells can synthesise ACh.

The experiments with agonists and antagonists suggest that the motoneurons have three kinds of ACh receptor. The properties of these receptors seem very similar to those described by Kehoe (1972) in Aplysia.

It is generally held that a particular neuron can only respond to a particular neurotransmitter in one way. It is therefore unlikely that the N2 and N3 neurons release ACh as their transmitter becaue of their opposite effects in some motoneurons (e.g. 3, 7 cells, Table 1).

ACKNOWLEDGEMENT

We should like to thank the Nuffield Foundation for their kind support.

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SEROTONIN-IMMUNOREACTIVE NEURONS IN THE CNS OF HELIX AND LYMNAEA

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Serotonin (5-HT) has been shown to be one of the major neurotransmitter candidates in the gastropod nervous system, involved both in central and in peripheral regulatory processes (Gerschenfeld, 1973; S.-Rózsa, 1984; Walker, 1986). High concentrations of 5-HT in the central nervous system (CNS) of gastropods has also been detected (Gerschenfeld, 1973; Osborne, 1982; S.-Rózsa, 1984). Parallel with physiological and biochemical investigations, the neuronal localization of 5-HT has been demonstrated in several gastropod species, using both fluorescence histochemistry (Sakharov and Zs.-Nagy, 1968; Sedden et al., 1968; Audesirk, 1985; Salimova et al., 1987) and immunocytochemistry (Ono and McCaman, 1983; Croll, 1987; Croll and Lo, 1986; Hernádi et al., 1988).

No detailed description on the distribution of 5-HT immunoreactive (5-HTi) neurons has until now been presented. The application of the highly specific method of immunocytochemistry seems to be indispensable for the precise mapping of 5-HTergic neurons in the CNS of both the Stylommatophran Helix and the Basommatophoran Lymnaea, since they are widely used as model systems and are important for comparative neurobiological studies. Therefore, the aim of our work was to present a mapping and comparison of the distribution of 5-HTi neurons in the CNS of both species, thus providing a morphological basis of further analysis of 5-HTergic regulatory processes.

For 5-HT immunocytochemistry the circumoesophageal ganglion complex of Helix pomatia L. and Lymnaea stagnalis L. was fixed in 4% paraformaldehyde diluted to 0.1 M phosphate buffer. After

fixation the ganglia were thoroughly washed, part of them de-sheated, and then processed for whole-mount 5-HT immunocytochemistry. For immunostaining 5-HT antiserum (Immunonuclear Corp., Stillwater, USA) was used in the following dilutions: 1:1000, 1:3000, 1:5000 and 1:8000. For the visualization of the 5-HTi elements Sternberger's PAP-method (Sternberger 1979) or an avidinbiotin method (Hsu 1981) was applied.

Specificity of the 5-HT antiserum has earlier been demonstrated (Ono and McCaman, 1983; Croll, 1987). Method specificity was controlled by replacing the primary antiserum by normal rabbit serum. No immunostaining could be observed after this control experiment.

5-HTi neurons can be observed in all parts of the CNS of both Helix and Lymnaea, except the pleural and buccal ganglia (Figs 1-5). Occasionally three 5-HTi small neurons could be seen in the buccal ganglia of Lymnaea, but the strong background immunoreactivity usually did not permit the localization of these neurons (Fig. 5a, question mark). The majority of the 5-HTi neurons

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Fig. 1. 5-HT immunoreactive neurons in the cerebral ganglia of Helix (a) and Lymnaea (b). MGC - metacerebral giant neuron, arrow - small-sized immunoreactive cell bodies. Bar represents 50 µm.

Fig. 2. 5-HT immunoreactive neurons (arrows) along the mediolateral margin of the pedal ganglia of Helix (a) and Lymnaea (b). Open arrow - immunostained single neuron with initial axon segment on the dorsal surface of the pedal ganglion of Helix. Bar represents 50 µm.

Fig. 3. 5-HT immunoreactive surface fibres of different diameter (curved arrows and open arrows) in the neural sheath of the cerebral ganglion. NC - nerve cells in the mesocerebrum, cc - cerebral commissure. Bar represents 20 µm.

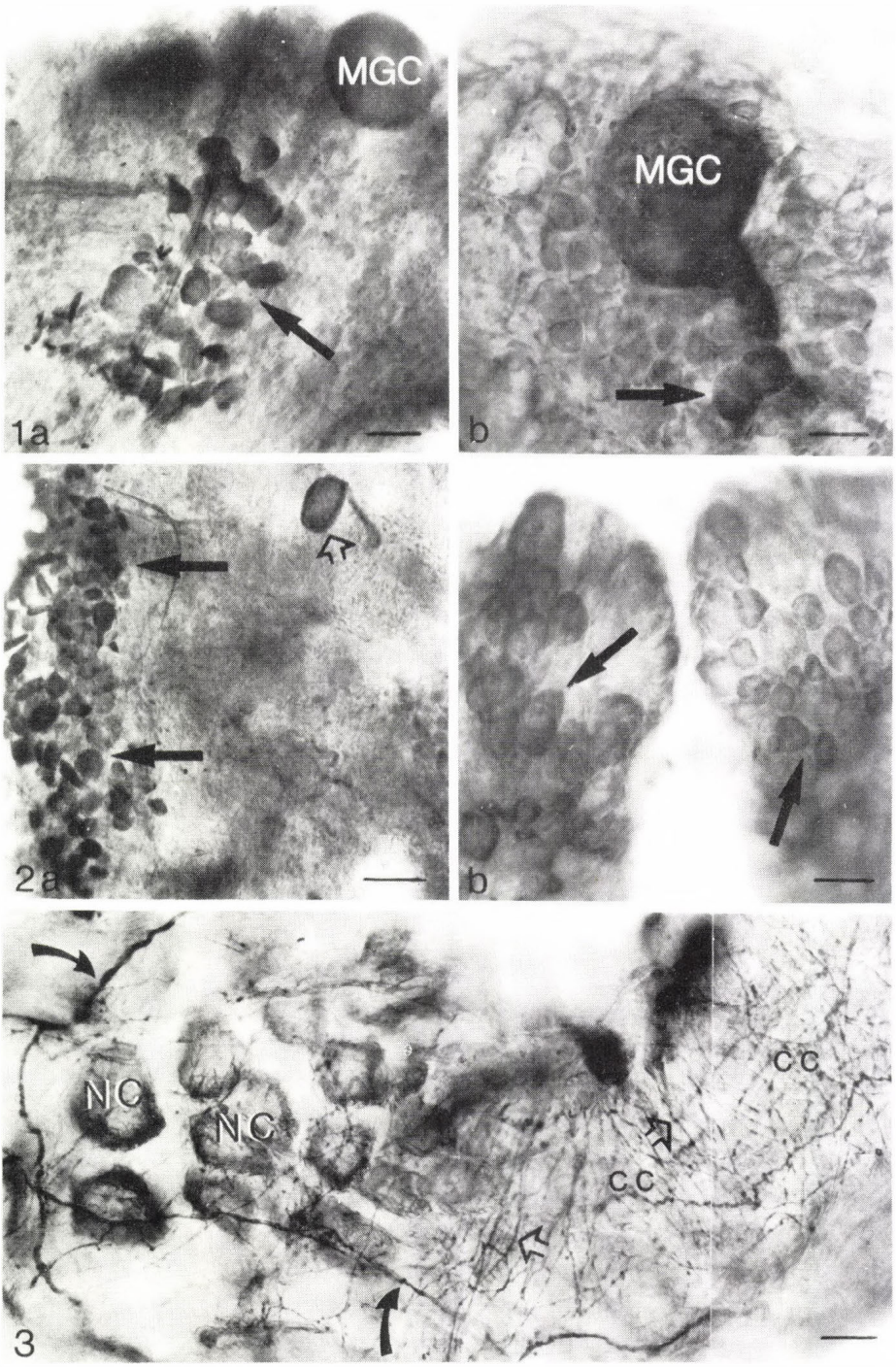
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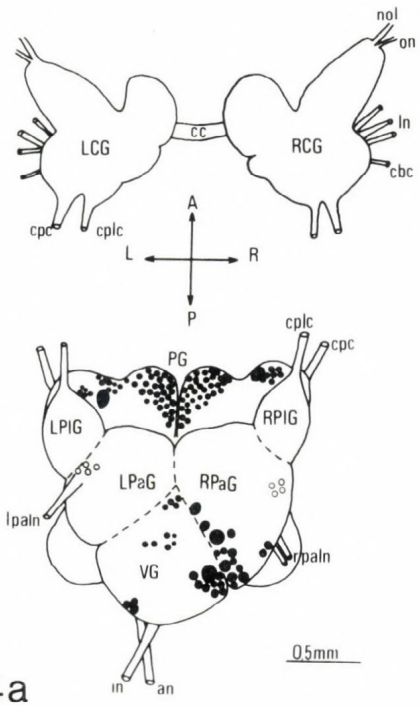
Fig. 4. Schematic representation of the distribution of 5-HT immunoreactive neurons in the central nervous system of Helix pomatia. (a) Dorsal, (b) ventral surface. Abbreviations: RCG – right cerebral ganglion, LCG – left cerebral ganglion, PG – pedal ganglion, RPG – right pedal ganglion, LPG – left pedal ganglion, RPlG – right pleural ganglion, LPlG – left pleural ganglion, RPaG – right parietal ganglion, LPaG – left parietal ganglion, VG – visceral ganglion, cc – cerebral commissure, nol – olfactory nerve, on – optic nerve, ln – labial nerve, cbc – cerebro-buccal connective, cpc – cerebro-pedal connective, cplc – cerebro-pleural connective, pn – pedal nerve, rpaln – right pallial nerve, plaln – left pallial nerve, in – intestinal nerve, an – anal nerve, MGC – metacerebral giant cell.

Fig. 5. Schematic representation of the distribution of 5-HT immunoreactive neurons in the central nervous system of Lymnaea stagnalis. (a) Dorsal, (b) ventral surface. The cerebral commissure was cut and the brain was spread out displaying the ventral surface of the cerebral ganglia in (a) and the dorsal surface in (b). Abbreviations: dbn, lbn, vbn, pbn – dorso-, latero-, ventro-, postbuccal nerve, cbc – cerebro-buccal connective, sln, mln – superior, median lip nerve, spn, mpn, ipn – superior, median, inferior pedal nerve, lpan, erpan, irpan – left, external, right internal, right parietal nerve, mcn – median columellar nerve, an – anal nerve, in – intestinal nerve, gn – genital nerve. Further abbreviations see in Fig. 4.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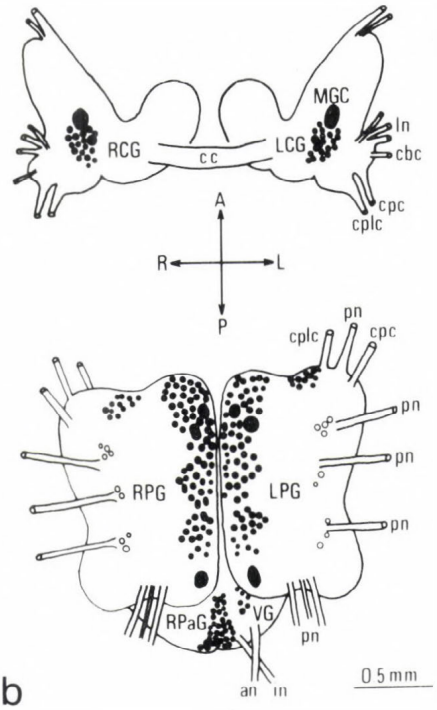
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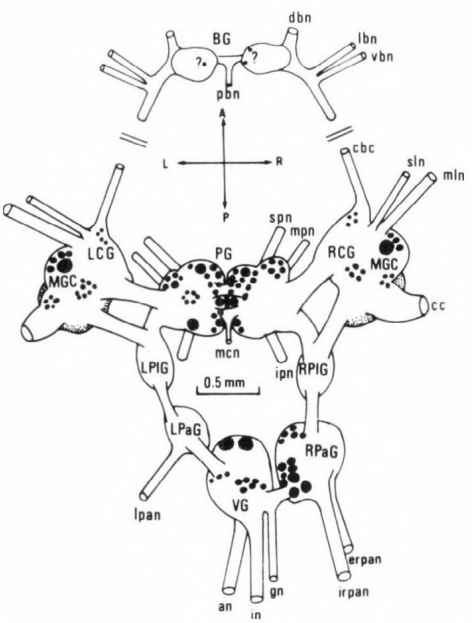
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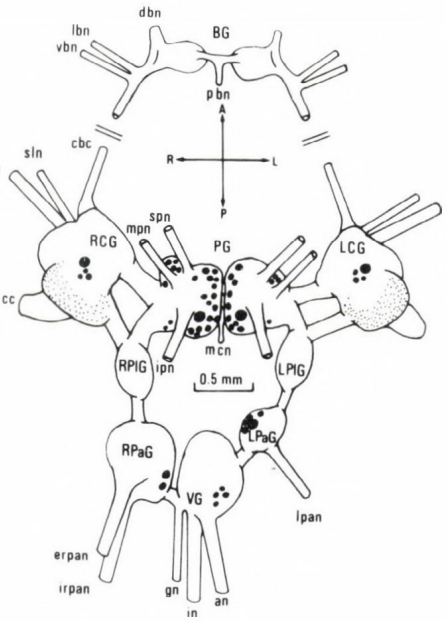
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are concentrated in the cerebral, pedal and viscero-parietal ganglia. Even in these ganglia, the 5-HTi neurons are not distributed evenly, but they occur mostly in groups. In the cerebral ganglia of Helix 5-HTi neurons are seen only on the ventral surface; here, the MGC and around it a cluster of smaller-size (2530 µm) neurons are immunolabelled (Figs 1, 4). In Lymnaea, 5-HTi neurons occur also on the dorsal surface: one large-size (80 to 100 µm) and several smaller-size (30-50 µm) neurons can be seen (Figs 1b, 5). On the dorsal surface of the viscero-parietal ganglionic complex of Helix typical 5-HTi cell clusters can be found along the borderline of the visceral and right parietal ganglia, while on the ventral surface immunostained neuronal somata of variable size are located at the caudal end of the visceral ganglion (Fig. 4).

In Lymnaea, a group of larger (60 to 80 µm) 5-HTi neurons are found on the medio-dorsal surface of the right parietal ganglion, while a group of 5-HTi neurons appears to spread over to the medio-ventral surface. A further typical group of stained neurons can be seen on the dorsal surface of the visceral ganglion (Fig. 5). In both species, a small group of 5-HTi neurons can additionally be observed in the left parietal ganglion. The great majority of 5-HTi neurons are located in the pedal ganglia in both species. They are present both on the dorsal and ventral surface, and are first of all concentrated along the inner (medio) lateral surface of each ganglion (Figs 2, 4, 5).

In addition to the 5-HTi neurons, immunostained axons can consequently be seen in all of the peripheral nerve roots, central connectives and commissures. A well-developed network of varicose 5-HTi fibres can be seen in the innermost layer of the neural sheath of the Helix CNS (Fig. 3), including the peripheral nerve roots. This system of 5-HT surface fibres is less prominent in Lymnaea.

The distribution and approximate number of 5-HTi neurons in the CNS of Helix and Lymnaea are similar and reveal some common characteristics: i) all ganglia except the pleural ones contain 5-HTi neurons; ii) the majority of 5-HTi neurons are located in the pedal ganglia; iii) the 5-HTi neurons are mostly located

in cell groups (clusters). This type of distribution of 5-HTi neurons has also been described in other gastropod species, e.g., Aplysia (Ono and McCaman, 1983; Goldstein et al., 1984; Longley and Longley, 1986), Hermissenda (Croll, 1987), Litorina (Croll and Lo, 1986). Also, a similar distribution of 5-HT-containing neurons has been described in the CNS of Helix and Lymnaea, when applying fluorescence histochemistry (Audesirk, 1985) as well as the neurotoxic false transmitter, 5,6-dihydroxytryptamine labelling technique (S.-Rózsa et al., 1986), respectively. The central role of these clusters of 5-HTi neurons in Helix and Lymnaea CNS in 5-HTergic regulatory processes is suggested from this work and may serve as primary targets for future studies dealing with functional aspects of 5-HTergic neurotransmission.

At the same time, an interspecies difference is that more 5-HTi neurons are present in the cerebral ganglion of Lymnaea (both dorsal and ventral surface), than in that of Helix (only ventral surface). The much better developed 5-HTi surface fibre network in the ganglion sheath of Helix may be due, at least partly, to the much thicker sheath, compared to that of the Lymnaea nervous system. This extremely well developed 5-HTi fibre network in Helix ganglion sheath may refer to an additional role of 5-HT in regulatory processes: remote control and/or modulation of peripheral processes through a neurohumoral way, by releasing 5-HT directly to the haemolymph. The presence of similar 5-HT-(monoamine) containing surface fibre networks has also been reported in other gastropod species: Philine (Barber, 1982), Aplysia (Ono and McCaman, 1983; Goldstein et al., 1984), Cryptomphalus (Flores et al., 1986).

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ACTIONS OF THE CATCH-RELAXING PEPTIDE ON SOME MOLLUSCAN MUSCLES

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INTRODUCTION

The catch-relaxing peptide (CARP) is a novel neuropeptide isolated from the pedal ganglia of the bivalve mollusc Mytilus edulis (Hirata et al., 1987). The primary structure of CARP is H-Ala-Met-Pro-Met-Leu-Arg-Leu-NH_2. This peptide shows not only relaxing action on catch tension in the anterior byssus retractor muscle (ABRM) of the mussel at low concentrations but also some other actions on the ABRM and other molluscan muscles (Hirata et al., 1987).

In this paper, we describe more detailed actions of CARP on the ABRM of Mytilus, radula muscles of the prosobranch mollusc Rapana and cardiac muscles of some bivalve molluscs.

MATERIALS AND METHODS

The following molluscs were used in the present experiments: Mytilus edulis, Meretrix lusoria, Tapes japonica (bivalvia) and Rapana thomasiana (prosobranchia).

The methods of dissection, stimulating and recording from the ABRM bundles of Mytilus and from the radula muscle bundles of Rapana were essentially the same as those of Muneoka and Twarog (1977) and Kobayashi (1972), respectively. The methods of dissection and recording from the hearts of Mytilus, Meretrix and Tapes were essentially the same as those of Welsh and Taub (1948).

The physiological saline for the experiments using bivalve molluscs was artificial seawater (ASW) of the following composition: 445 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$ and 10 mM Tris-HCl; pH 7.6. The physiological saline for the experiments using the prosobranch mollusc Rapana was low-Mg^{2+} ASW (20 mM $MgCl_2$). The low-Mg^{2+} ASW was made by replacing a part of $MgCl_2$ in normal ASW with osmotically equivalent NaCl. In some experiments, high-K^+ ASWs (100 mM-K ASW and 400 mM-K ASW) were used. These solutions were made by replacing a part of NaCl in normal ASW with osmotically equivalent KCl.

RESULTS AND DISCUSSION

It has been reported that CARP relaxes catch tension of the ABRM of Mytilus at low concentrations. The threshold concentration of the peptide for the relaxation is 0.3-1.0 nM and full relaxation during 5 min application can be observed at 10-30 nM (Hirata et al., 1987). These results were confirmed in the present experiments (Fig. 1A).

It has also been reported that CARP modulates phasic contraction of the ABRM in response to repetitive electrical pulses (15 V, 3 ms, 10 Hz, for 5 s) of stimulation. In general, the peptide shows potentiating action at lower concentrations and inhibitory action at higher concentrations. The threshold concentration for the potentiation is 0.3-0.5 nM. On the other hand, the threshold concentration for the inhibition varies widely with preparation: in some muscles 5 nM of CARP is enough to exhibit the inhibitory action, but in some other muscles 50 nM or more is required (Hirata et al., 1987). These results were also confirmed in the present experiments (Fig. 1B).

When the ABRM was stimulated with repetitive electrical pulses (15 V, 3 ms, 10 Hz, for 5 s) at 90 s intervals and a dose of CARP (5-500 nM) was applied to the muscle during an interval, it was observed that the inhibitory effect of the peptide reached its maximum 2-10 min after application.

Contractions of the ABRM in response to ACh and the molluscan neuropeptide FMRFamide were inhibited by CARP (50 nM).

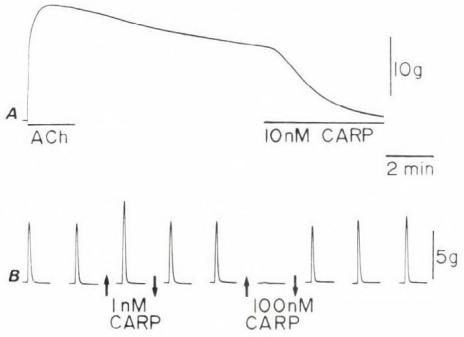

Fig. 1. Relaxation of catch tension (A) and modulation of phasic contraction (B) of the ABRM of Mytilus by CARP. Catch tension was produced by applying 10^{-4} M ACh for 2 min. Phasic contraction was evoked at 10 min intervals by applying repetitive electrical pulses (15 V, 3 ms, 10 Hz, for 5 s) of stimulation. Note that 1 nM CARP potentiated the phasic contraction but 100 nM CARP almost completely inhibited it.

FMRFamide contraction was more effectively inhibited than ACh contraction, though the degree of inhibition was found to vary considerably with preparation in both cases. In some muscles, ACh contraction was little affected by 50 nM CARP.

Contraction of the ABRM in response to 400 mM-K ASW was not inhibited by 50 nM CARP, but contraction by 100 mM-K ASW was inhibited a little by the peptide. Peak tension of the contraction was depressed to 80-90% of the control tension. Contraction in response to caffeine (20 mM) was also inhibited by 50 nM CARP. Its peak tension was depressed to 50-75% of the control tension.

In the hearts of Meretrix, Mytilus and Tapes, their activities were inhibited by CARP, though the threshold concentration for the inhibition was found to vary widely with animal species: the threshold concentration was about 5 nM in Meretrix (Fig. 2A), 1 nM in Mytilus and 50 nm in Tapes. The cardiac activity of the prosobranch mollusc Rapana is inhibited by the peptide but that of the pulmonate mollusc Achatina fulica is not affected by it even at 10^{-5} M (Kobayashi et al., unpublished).

It was found in Meretrix that the ACh antagonist benzoquinonium (10^{-5} M) did not affect the inhibitory action of CARP

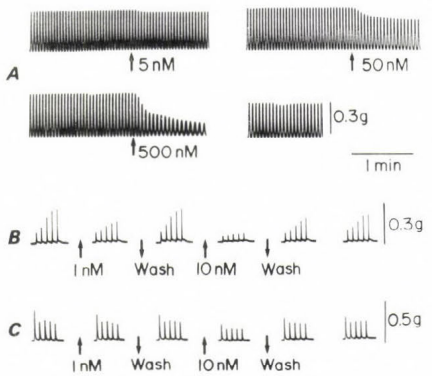

Fig. 2. Inhibition of cardiac activity (A) of Meretrix and twitch contractions of the radula protractor (B) and retractor (C) of Rapana by CARP. Between each record in A, the heart was washed with ASW for 10 min. In B and C, train of electrical pulses (15 V, 1 ms, 0.2 Hz, 5 pulses) of stimulation was applied at 10 min intervals.

(100 nM) on the cardiac activity, though the antagonist completely blocked the inhibitory action of ACh (1 nM).

In the radula protractor of Rapana, train-pattern twitch contractions in response to electrical pulses of stimulation (15 V, 1 ms, 0.2 Hz, 5 pulses) were depressed by CARP. The threshold concentration of the peptide for the inhibition was less than 1 nM (Fig. 2B). Train-pattern twitch contractions of the radula retractor were also inhibited by the peptide, though the retractor seemed to be less sensitive to the peptide than the protractor (Fig. 2C).

In the radula protractor and retractor muscles, both ACh and glutamate are capable of inducing contraction of the muscles (Kobayashi and Muneoka, 1980; Muneoka and Kobayashi, 1980). In the present experiments, it was found that both of the contractions of the muscles were inhibited by CARP (10 nM). Glutamate contraction was more effectively affected than ACh contraction. In these experiments, submaximal concentrations of glutamate (10^{-4} M) and ACh (10^{-5} M) were used to evoke contractions.

In addition to the relaxing action on the ABRM of Mytilus, CARP seems to have an inhibitory action on many molluscan

muscles, and the inhibition seems to be brought about by the direct action of the peptide on muscle fibres. The physiological action of CARP has not yet been clarified in any animal, but it can be speculated that the peptide plays and interesting role in molluscan neuromuscular systems.

ACKNOWLEDGEMENTS

We thank Dr. I. Kubota and Mr. N. Iwasawa for the gift of synthetic CARP. This work was supported by the Grant-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture of Japan.

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SOUND RESPONSES OF GIANT INTERNEURONS OF THE DECEREBRATED MALE CRICKET IN THE MOOD OF EVASION OR COPULATION

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While much effort has been made to understand the neural circuit responsible for a central pattern generator in invertebrates, few studies have been done on the relationship between neural activity and the animals' internal state: motivation, mood, or warming up. This is partly because of difficulty in training lower order animals and partly because of inconsistency in elicitation of the responses. The male cricket does not readily exhibit mating acts when it is restrained with the pronotum fixed to a holder.

The male cricket is known to exhibit copulatory behaviour even after decapitation (Huber 1955) or decerebration (Kitagaki and Sakai 1986). We found that such behaviour could be elicited more consistently in the decerebrated male than in intact males. We also found that this copulatory response could be modified by electrical stimulation. Thus, we examined the response to the same stimulus in two opposing behavioural states, that is, copulatory and evasive mood. First, ascending interneurons including giant interneurons in the terminal abdominal ganglion were investigated. Then, to determine the prerequisite for copulation augmentation some pharmacological agents were tested.

The male cricket Gryllus bimaculatus was held belly uppermost. Motoneuron activities were monitored through suction electrodes from the trochanter depressor and levator nerves innervating the hindleg. The physical stimulus for evoking behavioural response was applied intermittently to the periproct and cerci. The sensory interneuron response to a sound (dura-

tion 100 ms; 500 Hz) was recorded extracellularly with bipolar hook electrodes placed on the ventral nerve cord (VNC) in the abdomen. Electrical stimulation was delivered 20-30 times or the VNC at the neck (ESN) or the genitalia (ESG) in a pulse train (duration 1 ms; interval 2 ms) at 1 Hz. Octopamine was injected into a body cavity at a concentration of 10^{-4}.

Figure 1 shows schematic illustrations on sexual excitation change in the normal mating and the effects of electrical stimulation in the intact and decerebrated male. The sexual activity increases when it is paired with a female (♀) in the mating period and decreases rapidly after successful copulation (Non-excited) accompanied by spermatophore extrusion (SP-Ext) (Fig. 1, 1). If completion of copulation is prevented, the sexual activity increases considerably (Highly-excited) and then decreases during resting (Resting) and further decreases (Non-excited). ESG in the intact male (Fig. 1, 2) stops the sexual behaviour in the state that corresponds to "Resting" or "Non-excited". In the decerebrated male, ESN induced a "Highly-excited" state while ESG induced a "Low-excited" or "Non-excited" state (Fig. 1, 3).

Figure 2 shows an example of the experiment under the paradigm (Fig. 1, 3). The pattern of two motoneuron activities in response to a tactile stimulus indicates whether the response is evasive or copulatory. During copulatory mood, some of the giant neurons responded with a latency of 5-10 ms for 40 ms to sound stimulus (Fig. 3). After evasive mood was induced by ESG, the neuronal response was suppressed. The original response recovered when the copulatory mood was again induced by ESN. However, such a case was observed in only 15% of preparations so far. No case was found in which the neural response was in the reverse direction. In 85% of the males, the interneuron response was virtually the same during both behavioural states.

Octopamine augmented the copulation response similarly as ESN, and enhanced the giant interneuron response. The functional significance of this increased responsiveness during copulation mood remains enigmatic since the giant neuron is primarily involved in the escape system.

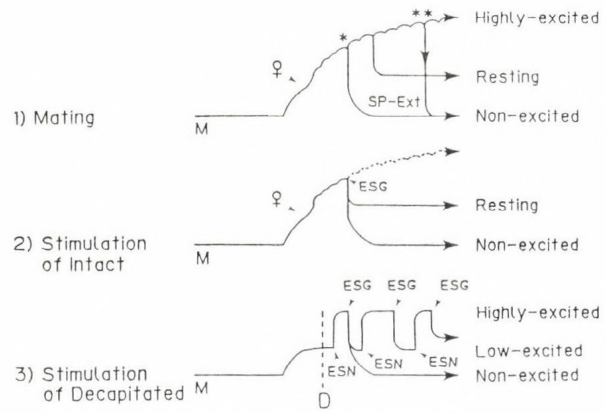

Fig. 1. Schematic diagrams showing changes by electrical stimulation in intact and decerebrated male cricket.

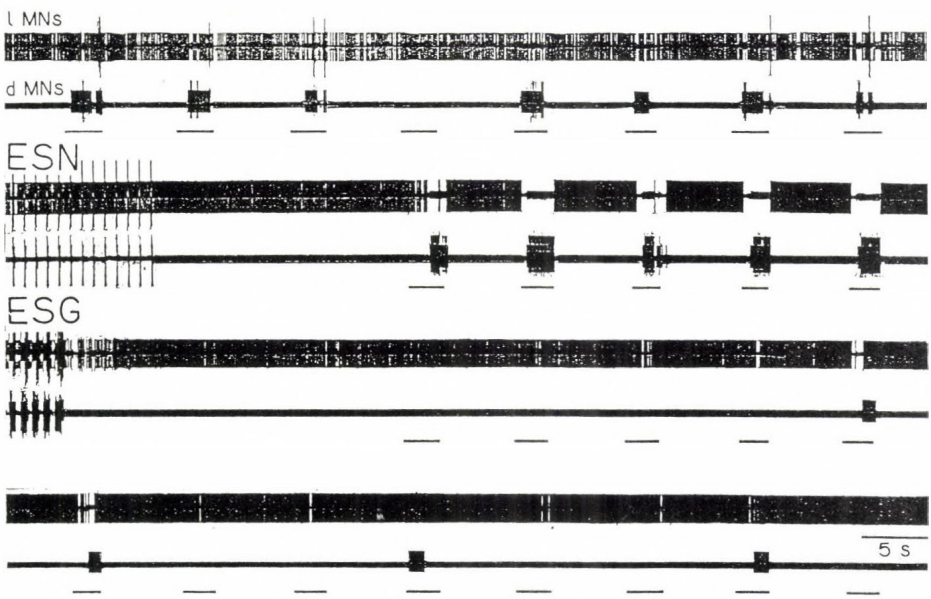

Fig. 2. Augmentation and suppression of the copulation response by electrical stimulation in the decerebrated male cricket. ESN: Stimulation of VNC at the neck. ESG: Stimulation of the genitalia. l MNs: Levator motoneurons. d MNs: Depressor motoneurons. Bar: Tactile stimulation on the periproct and cerci. ESN enhanced a copulatory response pattern while ESG suppressed it and induced an evasive response pattern.

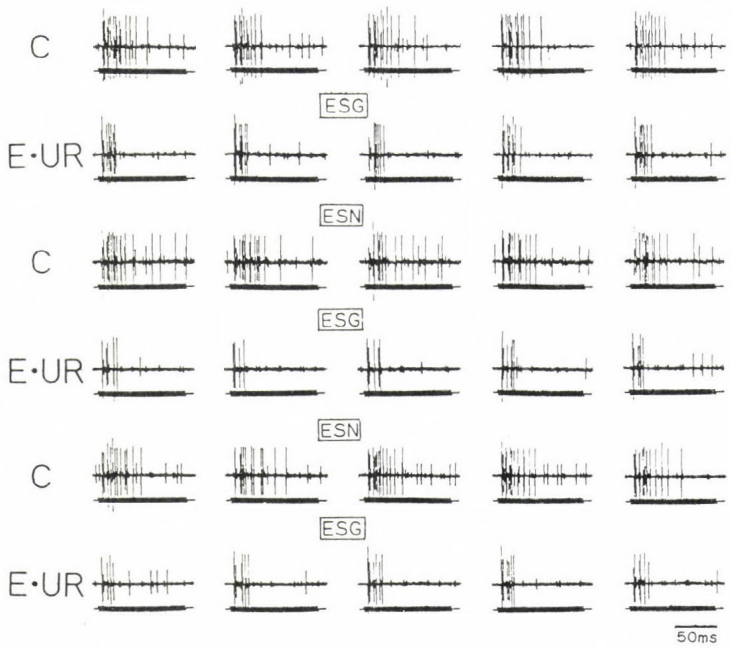

Fig. 3. Augmentation and suppression of the copulation response by electrical stimulation in decerebrated male cricket.

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4-AMINOPYRIDINE BLOCKS DIFFERENT CHOLINERGIC RECEPTORS IN APLYSIA NEURONS DIRECTLY

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4-Aminopyridine (4-AP) is a classical blocker of voltage dependent potassium channels in different membranes. It has been demonstrated that in addition, 4-AP can reduce and block acetylcholine (ACh) induced currents; not only those carried by potassium (1) but also those carried by sodium and chloride ions (3,5). This paper describes new experiments which showed that 4-AP acts directly on ACh receptors in Aplysia neurons.

An example of this action of 4-AP is shown in Figure 1. By recording from cell R-15 under current clamp conditions we compared changes of the amplitudes of excitatory post-synaptic potentials (EPSPs, column 1) evoked by electrical stimulation of a specific nerve fiber with those potentials evoked by local application of ACh (iontophoresis or pressure ejection; column 2). After adding 4-AP in a concentration of 100 μM/l to the bath solution (artificial sea water), the synaptically evoked EPSPs are enhanced by 45%, but under the same conditions the ACh responses are reduced by nearly 50% (B1 & B2). An increase in EPSP amplitude can be observed with concentrations up to 600 μM/l of 4-AP. At higher concentrations of 4-AP the synaptically evoked potential is strongly reduced, e.g. under 1 mM/l 4-AP shown in C1. Under these conditions even the increase of the duration of the ACh application from 1 sec to 1.5 sec causes only a very small increase of the ACh response (C2). Quite often these effects are completely reversible. After more than one hour wash the EPSPs and the ACh responses are again slightly increased.

The discrepancy between the effects of 4-AP on the synaptically and the ACh evoked potentials can be explained by a fascillatory effect of 4-AP on presynaptic mechanisms (2,6) which far outways the blocking effects of the same drug on post-synaptic receptors at concentrations up to 600 μM/l. We found that the cholinergic, chloride dependent IPSP of cell L-11 showed

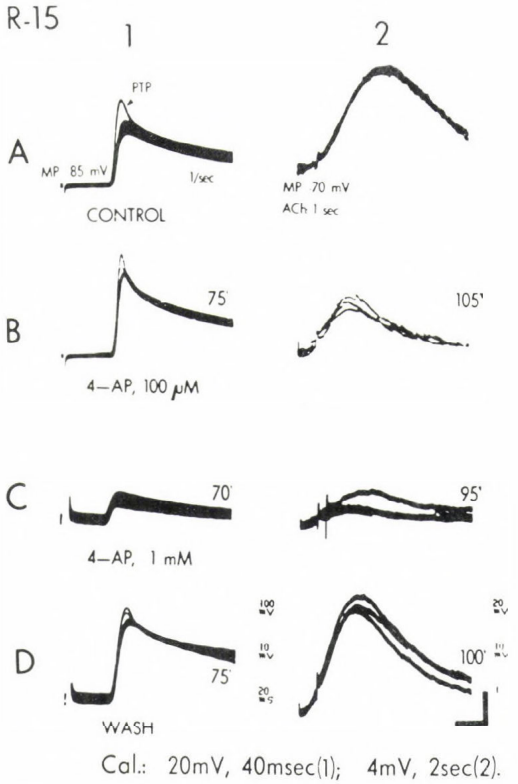

Fig. 1: 100 superimposed EPSPs (1c/sec) and one PTP-EPSP (30 sec after the tetanus, arrow) in column 1 and 5 superimposed responses to ACh (1 sec pulses; column 2) in control, during 2 different concentrations of 4-AP and during wash. Discrepancy between changes of the EPSPs and ACh responses under 100 μM/l 4-AP (line B).

an enhanced amplitude under 4-AP concentrations up to 1 mM/l. The early, chloride dependent ACh response of the medial cells in the pleural ganglia together with the late potassium dependent ACh response of the same cells were both reduced by approx. 80% by 1 mM/l 4-AP (3). The potential most sensitive to the application of 4-AP was the non-cholinergic inhibition of long duration which can be synaptically evoked in cell R-15. This ILD was blocked by 4-AP at a concentration of 100 μM/l (3).

Reductions in sodium currents evoked by ACh in cell R-15 or RB cells, caused by 4-AP, were analyzed under two needle voltage clamp conditions. In the Lineweaver-Burk plot these data suggest that 4-AP is a non-competitive blocker of ACh on those receptors (3).

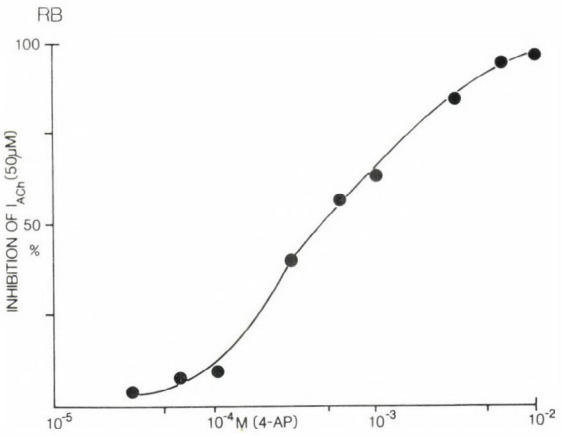

Fig. 2: Percentage of reduction of an ACh (50 µM/l) induced sodium current in a RB cell caused by 4-AP in concentrations of 30 µM/l to 10 mM/l. K_m of approx. 400 µM/l of 4-AP.

The experiments described so far were done in the visceral ganglion with an intact or only slightly opened capsula. To guarantee full action of 4-AP we pretreated the preparation for a longer duration (30-60 min), because after short pretreatments the effect of 4-AP could vary. To overcome those problems and to get insight into the time course of action of 4-AP we reinvestigated the action of 4-AP by using the so-called "concentration clamp" method introduced by Slater and Carpenter (7). We used a two-needle clamp amplifier and recorded from a group of completely dissected neurons. Rapid exchange of the perfusion solution within 2-5 msec was achieved by a perfusion tube focused directly on the neurons. The ACh test solution was of 50 µM/l concentration. To this ACh solution, 4-AP was added in different concentrations, i.e. the neurons had no pretreatment of 4-AP at all. The result obtained from the cells R-15, RB group and L-11 demonstrated that 4-AP acts directly and immediately on the ACh receptors without an intermediate step. The sodium dependent ACh responses were about 10 times more sensitive to 4-AP than the chloride dependent responses of cell L-11. Figure 2 shows the result of such an experiment.

The 4-AP concentration that caused 50% reduction of the sodium currents, K_m, was between 250 and 400 µM/l in different experiments. In contrast, the K_m for the reduction of the chloride current of cell L-11 was around 4.5 mM/l. These data fit with those obtained under current clamp conditions in

respect to changes of PSPs and ACh responses induced by bath application of 4-AP. In contrast to the reduction of chloride currents seen in Aplysia, Ikemoto et al. (4) observed in unidentified isolated and perfused Helix neurons that 4-AP itself in millimolar concentrations generates chloride currents and that those chloride currents can potentiate with ACh evoked chloride currents.

For Aplysia neurons one can conclude that the receptor with the highest sensitivity for a blockade by 4-AP are those for cholinergic and non-cholinergic slow potassium responses (1,3). While sodium dependent responses are blocked by micromolar concentrations of 4-AP, the chloride responses will be reduced only by 4-AP in the millimolar range. For the sodium response we could demonstrate that 4-AP is a non-competitive blocker for ACh receptors.

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COMPARATIVE STUDIES ON THE EFFECT OF ACh AND GABA ON THE IDENTIFIED NEURONS OF PLANORBARIUS CORNEUS

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The effects of gamma-aminobutyric acid (GABA) and acetylcholine (ACh) on the same identified neurons from pedal and visceral ganglia were compared. The experiments were carried out on the neurons in the ganglia and on the completely isolated neurons. Microelectrodes were filled by potassium sulphate (0.5 M/l) or potassium chloride (2.5 M/l).

Twenty-five identified neurons responded to ACh (Gapon 1983). Of these, 21 neurons were found to have two types of cholinoreceptors; similar acetylcholine receptors of LPed-2, LPed-3 and LPed-4 neurons (Ger et al. 1977). Nineteen neurons responded to GABA. Some cells were depolarized by GABA, others were hyperpolarized, and two neurons gave biphasic response. The effects of GABA and ACh were found to be similar in the same neurons.

The sensitivity of the neurons to ACh was usually higher than the sensitivity to GABA, with the exception of the LPed-9 neuron.

The GABA response of the LPed-2 neuron was investigated. The LPed-2 neuron is one of the largest neurons and the nature of its response has been studied (Zeimal et al. 1975, Kobzar 1979). The reversal potentials for ACh and GABA were found to be equal (about -25 mV). In the several experiments the amplitude of response to ACh was similar to that of GABA. The equal magnitude of the reversal potential gives proof of the same ionic nature of the ACh and GABA reaction.

In two experiments microelectrodes were filled with K_2SO_4.

The value of the responses to GABA and ACh was compared to the level of membrane potential on the seven neurons:

LPed-2	E_{ACh} = -25 mV,	E_{GABA} = -24 mV
LPed-8	E_{ACh} = -30 mV,	E_{GABA} = -29 mV
LPed-9	E_{ACh} = -23 mV,	E_{GABA} = -30 mV
V - 7	E_{ACh} = -17 mV (D-c.)	E_{GABA} = -17 mV (D component)
	E_{ACh} = -65 mV (H-c.)	E_{GABA} = -65 mV (H component)
V - 8	E_{ACh} = -80 mV,	E_{GABA} = -80 mV
V - 5	E_{ACh} = -40 mV,	E_{GABA} = -40 mV
LPed-4	E_{ACh} = -47 mV,	E_{GABA} = -50 mV

Reversal potentials for GABA and ACh were identical in all studied neurons. The reversal potential for hyperpolarizing response or H component of GABA response as well as the K^+-dependent ACh effect were found at -80 mV. The reversal potential for depolarizing effect or D component of GABA response was from -20 to -30 mV, corresponding to the Cl-dependent effect of ACh. Presumably both GABA and ACh can alter the permeability of the membrane for K^+ and Cl^- ions alike. The biphasic nature of the responses suggests two types of permeability changes in some neurons.

To show the existence of the same or separated receptors in GABA and ACh effects, the inhibitors of their receptors, e.g. tubocurarine (d-TC) and picrotoxin were used. The depolarizing response of the LPed-2 cell to ACh was blocked by d-TC while the GABA response remained unaltered. On the contrary, picrotoxin blocked the depolarizing response to GABA and failed to modify the ACh response. There are GABA and ACh receptors in the soma of the LPed-2 cell.

More direct evidences of the existence of independent GABA and ACh receptors were found in isolated neurons. The neuron V-7 gives biphasic ACh and GABA responses. Picrotoxin blocked the depolarizing component of the GABA response, while this component of ACh response remained unchanged. d-TC blocks vice versa the depolarizing component of the ACh response and has no influence on the depolarizing GABA component.

These results show that the same permeability changes evoked by GABA and ACh are connected with different receptors.

To study the effects of GABA and ACh furosemide was used, blocking transport of Cl^- ions in various systems. Furosemide blocked increase of Cl^- conductance evoked by application of ACh and suberyldicholine (D-6) as well as GABA. Furosemide decreased the amplitude of the GABA response and conductance (Fig. 1). After treatment with furosemide ($2x10^{-4}$ g/l) the con-

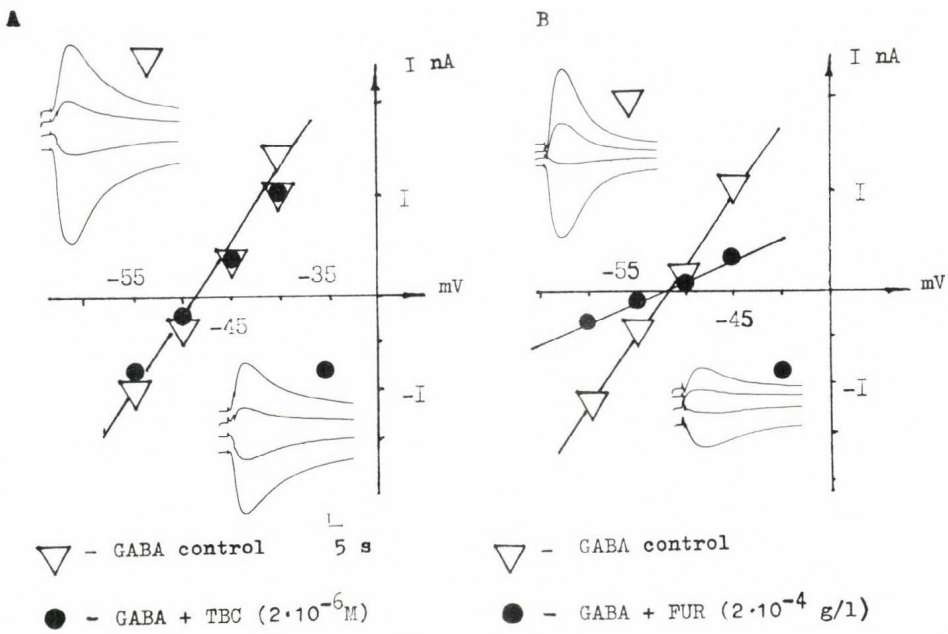

Fig. 1. Effects of furosemide and d-TC on responses produced by ionophoretic application of GABA of LPed-2 neuron.

ductance was 2.7 times lower. Shift of the reversal potential was not observed. d-TC had no effect on the conductance. This proves that the GABA effect is not connected with the cholinoreceptor activation.

D-6 activates receptors controlling chloride conductance. The amplitude of response to D-6 and the conductance are decreased by d-TC and furosemide. In the presence of furosemide the response is elevated maximum 1.5 times more slowly. The time constant of decay increased 1.95-fold. d-TC did not show similar effect on the response. Therefore, the mechanism of the

furosemide effect differs from that of the d-TC effects. ACh-activated (and dioxalane-activated) increase in K^+ conductance as well as ACh-activated Na^+ conductance was found to be unaltered. In Fig. 2A biphasic response of LPed-3 to ACh is

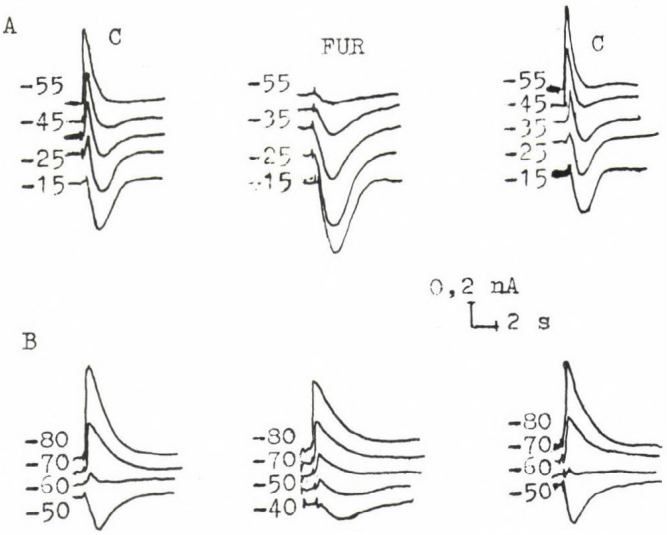

Fig. 2. Lack of blocking effect of furosemide on K^+-dependent responses to dioxalane F-2268 of LPed-3 neuron (A) and on Na^+-dependent responses to ACh of LPed-2 neuron (B).

shown. Inward current (more rapid) is caused by Cl ions, outward current (slow) by K^+ ions (Ger and Zeimal 1976). In the presence of furosemide the rapid component of the ACh response was blocked, while the slow component remained unaltered. The response of LPed-2 (Fig. 2B) to ACh consists of two components, one being Na^+-dependent, the other being chloride-dependent (Ger et al. 1980). Furosemide shifts the reversal potential towards E_{Na} blocking of chloride conductance (Fig. 2B).

Unlike d-TC and picrotoxin, furosemide appears to act directly upon ion channels but does not alter the receptors themselves. Neither Na^+-, nor K^+-dependent responses induced by cholinoreceptor activation were changed by furosemide. Furosemide was found to diminish both the amplitude and the rate of Cl-dependent responses. The lack of selectivity to ACh or

GABA suggests that furosemide blocks the Cl channels of the chemoceptive membrane, which are probably common to ACh and GABA, and does not affect their receptors.

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SYNAPTIC POTENTIALS IN THE HEARTS OF MOLLUSCS

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INTRODUCTION

The molluscan heart is regarded to be typically myogenic. In the myocardial cells the intracellular or quasi-intracellular recording shows a distinct slow diastolic depolarization until a threshold is reached, then a pacemaker-like action potential is triggered (Jones 1983).

The nervous system provides an extracardial regulation using inhibitory and excitatory neurotransmitters (S.-Rózsa 1972). In experiments on some gastropod heart preparations with intact innervation "little oscillations of potential" have been shown connecting to the nerve regulation (Zhuravlev et al. 1985). An analysis proved that they are postsynaptic potentials (PSPs). It has been found that PSPs can regulate and coordinate the contractions of heart muscle.

RESULTS

The experiments were performed at the heart preparations of various gastropod species: Helix sp., Achatina fulica, Lymnaea sp. Electrograms of the heart have been recorded by extracellular silver wire microelectrodes or flexible suction electrodes filled with 0.2 M KCl. The intracellular activity of 1-2 neurons was recorded with conventional microelectrophysiological method and the cell membrane was depolarized through microelectrode. A spontaneous synaptic inflow consisting of discrete

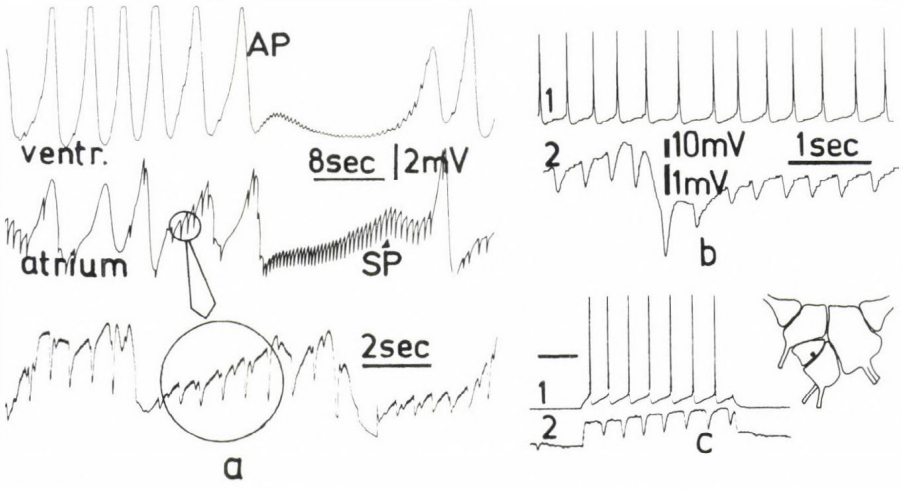

Fig. 1. Synaptic potentials in the heart of Helix pomatia L.
a - Traces of simultaneous recordings from the ventricle and auricle by suction electrodes. AP - action potentials, SP - synaptic potentials. Lower trace: activity in the auricle recorded with higher amplification.
b - Activity of the "fast inhibitory" neuron (1) from the visceral ganglion, and IPSPs in the auricle (2).
c - IPSPs in the auricle after stimulation of the cell.

IPSPs and EPSPs of 150-200 ms duration (Fig. 1a) was found in each preparation. The PSPs in different regions of the heart could be generated both synchronously and asynchronously, with a mean frequency of 2-3 cps. In the auricle and ventricle inhibitory synaptic potentials were found to predominate. Occasionally, at the same area of the myocardium 2-3 types of PSPs can appear simultaneously emphasizing different sources of their origin.

In the Helix and Achatina hearts EPSPs can mainly be recorded from the ventricle near the aorta and from the atrio-ventricular valve while Lymnaea heart had a more homogeneous distribution of EPSPs. The IPSPs are recorded all over the myocardium.

The activity of well-known cardioregulatory neurons (S.-Rózsa and Zhuravlev 1981) has no correlation with the above synaptic potentials. These neurons alter the frequency of the

heart beats by aid of slow membrane potential shifts, or "slow PSPs". Slow PSPs with a duration of a few seconds can be recorded from both the resting heart preparations and from those having initially low frequency beating. The stimulation of the intestinal nerve evoked inhibitory and excitatory slow and fast PSPs, and reciprocal effects of the cell stimulation on the auricle and on the ventricle have also been shown. Methysergide blocked the synaptic transmission from the "slow" cardio-excitatory neurons to the heart.

In a visceral ganglion of Helix sp., cells are identified and are shown to produce fast IPSPs in the auricle with 180-200 ms delay (Fig. 1b). Being stimulated or firing spontaneously these neurons can effectively inhibit the auricle. D-tubocurarine blocked IPSPs in the heart. A pair of giant neurons in a medio-rostral area of parietal ganglions induce EPSPs in the ventricle.

In Lymnaea heart one single stimulus to an intestinal or left pallial nerve trunk evokes an extrasystole without changing the frequency of heart beat. Sometimes the single stimulation induces immediately or with 10-20 s delay rhythmic heart beats, lasting for an hour. In this case simultaneously with the single heart contractions burst patterns were recorded from the left pallial nerve (Fig. 2a).

The burst pattern recorded from the left pallial nerve induced an EPSPs burst in the auricle and simultaneously an IPSPs burst in the ventricle (note that in this experiment no contraction appeared in the ventricle). At the beginning the bursts appear asynchronously with the systole, but after 30-40 s they become synchronous and the bursts appear just before the auricle contracts (Fig. 2b). At the same time IPSPs appeared during hyperpolarization of the myocardium. Thus, for a long time (one hour) the myocardium having an endogenous pacemaker behaves as a somatic muscle: the contraction follows the EPSPs bursts. The central generator of the rhythmicity has not been identified, although the neurons in the right parietal ganglion are known to be in connection with them. These interneurons generate EPSPs or AP in their somata before the systole, but it has no effect on the heart.

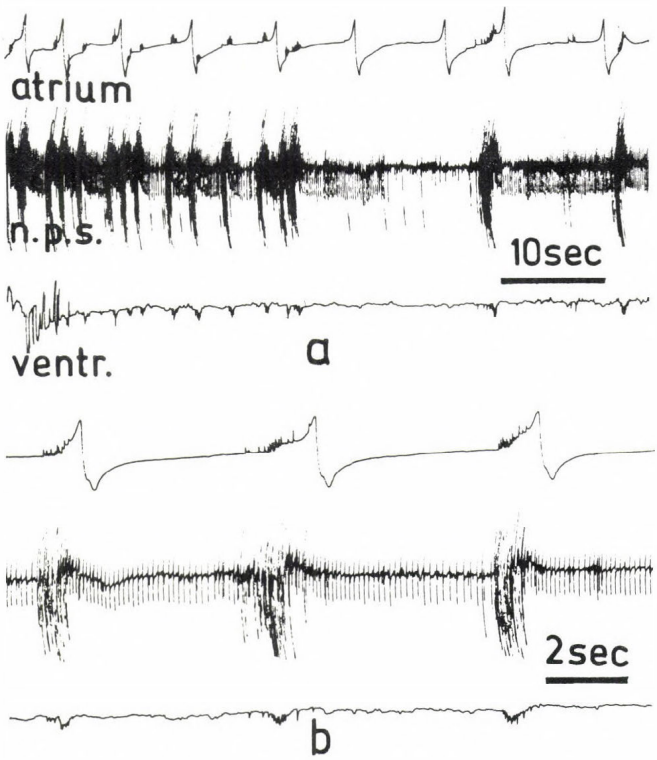

Fig. 2. Synchronization of the neuronal discharge and contractions of the auricle in Lymnaea stagnalis. a - Generation of burst pattern after short stimulation of the n. intestinalis. Before stimulation no correlation was observed between the contractions and bursts of the n. pallialis sinister (n.p.s.). b - Activity 40 s after the fragment "a" has been recorded. AC - amplifier with a time constant of 0.3 s has been used.

The results suggest several types of regulatory system in a gastropod heart: there are slow and fast excitatory and inhibitory postsynaptic potentials. There is no conduction between the ventricle and auricle of the heart in Helix, although the heart normally contracts coordinately (Ripplinger 1957). Fast PSPs were found to be much shorter than the interval between the contractions, so they may act in an opposite phase with the pacemakers. It is suggested that a slow system may regulate a frequency of the heart beats as a whole while a fast system

regulates the frequency of contraction of the ventricle and auricle separately, in order to coordinate their contractions.

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ON THE ROLE OF GLIAL CELLS IN DUAL SECRETORY DYNAMICS OF THE CAUDODORSAL CELLS OF LYMNAEA STAGNALIS

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The cerebral Caudodorsal Cells (CDC) of the pond snail *Lymnaea stagnalis* control egg laying and egg-laying behaviour by releasing various peptides from two types of release site: 1. into the haemolymph, from neurohaemal axon terminals located in the outer compartment of the cerebral commissure, and 2. into the intercellular space of the central nervous system (CNS) from nonsynaptic release sites of axon collaterals in the inner compartment of the commissure. It is assumed that peptides released into the haemolymph act upon peripheral targets (gonad, accessory sex glands) whereas peptides released into the CNS control central neurons that are involved in, e.g., locomotory and posture changes during egg laying. In this way the CDC control the various stages of overt and covert egg-laying behaviour of *L. stagnalis* in a well-timed and coordinated fashion (Roubos et al., this volume).

In the past decade the dynamics of neurohaemal release have received much attention (for review see Roubos, 1984). Neurohaemal release of egg-laying hormone (CDCH) and of other CDC-peptides particularly occurs during the active state, a ca. 1 hour lasting period of high electrical activity of the CDC; during electrical silence CDCH-release takes place at a low level only. Recently, attention has been focused at the dynamics of CDCH-release from the collaterals. The nonsynaptic release sites lack the morphological specializations characteristic of classical synapses but reveal exocytotic release of the contents of secretory granules. These sites are considered to be the morphological correlates of nonsynaptic (paracrine, hormone-like) communication. It is assumed that the peptides released from the collaterals diffuse throughout the inner compartment before acting upon their targets, which possess the appropriate receptors (Schmidt and Roubos, 1987a,b). In this way the CDC might control various neurons of different type and location, without having structural (axonal) interconnections.

Quantitative electron microscopy of exocytosis activity has indicated that CDCH-release from the collaterals particularly occurs during electrical silence and is low during the active state, i.e. a reverse situation as compared to neurohaemal CDCH-release (Schmidt and Roubos, 1987b; Roubos et al., this volume). (The mechanism by which the CDC are capable of releasing peptides from different release sites with different intensities is not known.)

Obviously, this duality of CDC secretory dynamics can only be of physiological significance if the concentrations of CDCH in the inner and outer compartment are controlled largely independently from each other. In this communication we present evidence that 1. such different concentrations of CDCH in the inner and outer compartments do exist and 2. this concentration difference is maintained due to the presence of the glial sheath which separates the inner and outer compartment and functions as a chemical ("blood-brain") barrier (cf. Schmidt and Roubos, 1987a).

The glial sheath consists of various (up to 10) adjacent layers of glial cells, which processes are interconnected by extensive desmosomes. The sheath is continuous with the glial cell layer that covers the cerebral ganglia. During *in vitro* incubation of CNS in a snail Ringer solution containing 0.5% trypan blue, cells of the glial sheath actively take up this dye. Since no trypan blue was found in the inner compartment, either in neuronal elements or in, dispersely occurring, glial cells, the sheath apparently blocks the penetration of the dye into the inner compartment. This conclusion is supported by ultrastructural studies of the fate of protein-A/gold particles (PAG) applied to the cerebral ganglia either by immersion of the CNS or by microinjection into the inner compartment. Two hours after immersion, uptake of PAG was observed in cells of the sheath; PAG was present in small (endocytotic?) and in large vesicular structures that possibly represented secondary lysosomes. No PAG was found in the inner compartment. One hour after injection of PAG into the inner compartment, the intercellular space as well as glial cells in this compartment revealed high amounts of PAG; hardly any PAG was found intra- or extracellularly in the outer compartment. Cells of the sheath, however, contained high amounts of PAG, present in small and large vesicular structures (Fig. 1).

In order to get an impression of the concentrations of CDCH in the inner and outer compartment quantitative immunoelectron microscopy with a polyclonal anti-$CDCH_{20-36}$ antibody was carried out using monodisperse goat anti-rabbit IgG-gold as the second antibody (cf. Roubos et al., 1987a), following tissue fixation according to the TAGO-method (Roubos and van der Wal-Divendal, 1980). (Sections had been etched previously with saturated metaperiodate.) The different dynamics of CDCH-release are clearly reflected by the numbers of immunogold particles above the intercellular space: during the active state immunoreactivity in the neurohaemal area is 5 times as high as in the inner compartment. A series of measurements at equidistant sites in each compartment shows that intercellular CDCH-immunoreactivity is fairly constant throughout each compartment (Fig. 2). Thus, the marked discontinuity in the degree of CDCH-immunoreactivity (i.e., of the concentration of CDCH) occurs at the transition between the inner and outer compartment: the glial sheath. This strongly indicates that the sheath functions as a barrier for CDCH and prevents the movement of CDCH from the outer into the inner compartment.

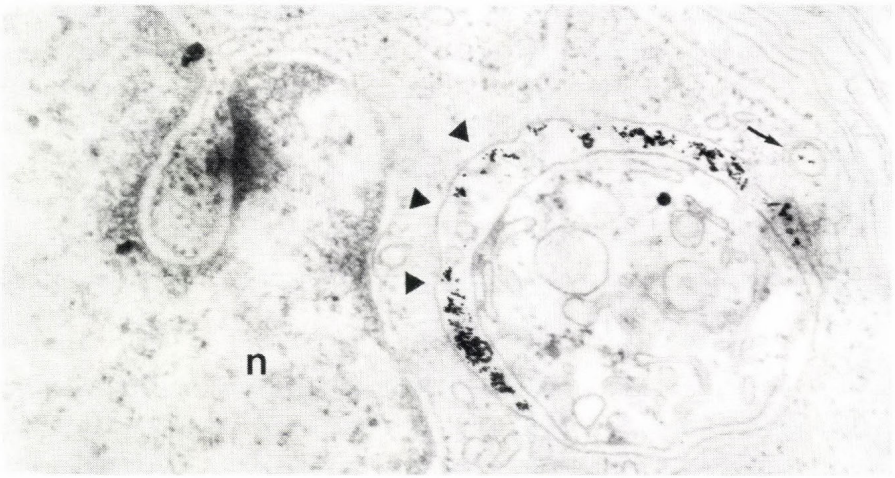

Fig. 1. One hour after PAG microinjection into inner compartment. Glial cell from sheath shows numerous PAG particles in small (arrow) and large vesicle (arrowheads). n, nucleus of glial cell. x60,000.

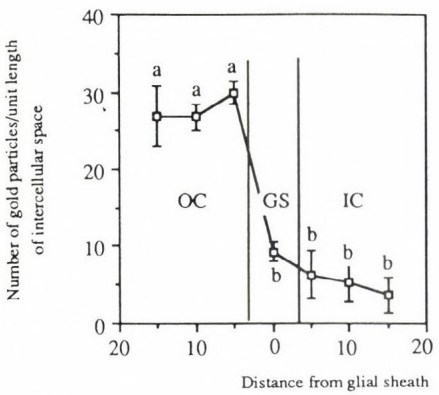

Fig. 2. Numbers (means ± S.E.M.) of immunogold particles (anti-CDCH)/unit length of intercellular space at equidistant sites in inner (IC) and outer (OC) compartment and in the glial sheath (GS), counted in cross-sections of cerebral commissures of snails with CDC in the active state. TAGO-method. Different characters indicate statistically significant differences ($p<0.01$).

CDCH-immunoreactivity is present not only in the secretory granules in the CDC axon terminals (Roubos et al., 1987a; this volume) but also in the cells of the glial sheath. Many of the immunogold particles are located over small, electron-lucent vesicles, suggesting endocytotic uptake of CDCH. This uptake seems to be specific of the sheath cells. Immunoreactivity in glial cells within the inner compartment does not exceed background labelling (counted above araldite areas outside the tissue).

Obviously, the glial sheath is not an absolute chemical barrier between the two compartments. Since the CNS has no vascular blood supply, various substances may have to

diffuse through the sheath. This is evident for tannic acid, which penetrates the sheath within a few minutes (E.D. Schmidt, unpublished results). Moreover, adjacent cells in the sheath are not always tightly connected by desmosomes but at some places reveal the regular intercellular space via which substances may be exchanged between the compartments. Therefore, it must be assumed that the glial sheath barrier acts selectively, preventing only particular substances from passing it. The significance of the uptake of various substances (PAG, trypan blue, CDCH) is obscure, but in the present context the possibility seems relevant that the glial cells particularly take up substances that travel along the intercellular space of the sheath, thus preventing them to reach another compartment.

In conclusion, the data indicate that the glial sheath functions as a barrier for CDCH (and, most likely, for other CDC-peptides). The glial cells seem to exert this function by blocking and, possibly, by ingesting the peptides. In this way the sheath may contribute to the control of peripheral and central targets by CDC-peptides released from the neurohaemal area and the collateral system, respectively.

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CLOSING REMARKS

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This has been a splendid international Symposium. Research work from many laboratories of no less than 14 countries has been presented and discussed in formal and informal sessions. The Hungarian Academy of Sciences must be congratulated for having such a marvelous institute - and such an effective director. Through his initiative and organizational talent, comparative neurobiology has been significantly stimulated and vitalized. This institute on the shores of Lake Balaton is indeed a showcase of Hungarian science and culture. All of us who have had the pleasure of participating in this Symposium have enjoyed the peaceful and relaxed atmosphere that permeates these exquisite buildings whose commodious facilities and lovely setting are so conductive to scientific discussions.

This meeting has dealt with three important topics of modern neurobiology: transmitters, modulators and receptors. The focus has been on the chemical processes involved in synaptic transmission, and on the chemical regulation of neuronal activity. The emphasis was on research on invertebrate animals, with species ranging from coelenterates to insects.

The reports were based on an impressive variety of research methods which, beside the classical techniques of electrophysiology, included immunocytochemistry as well as behavioural analyses. Most exciting, perhaps, is the new cytogenetic approach which provides insights into the mechanisms of gene expression involved in the production of certain neurotransmitters and/or neuromodulators.

The meeting made it clear that the new technologies now available to neurobiologists provide a wealth of new data for which established physiological concepts become increasingly inadequate. We loosely employ such terms as "synaptic transmission", "transmitter" or "modulator" but fail to adapt their definition to the knowledge gained through new research methods.

Some years ago, only a few neurotransmitters were recognized. It was thought that the nervous system of any animal species employed only a very small number of such transmitters. Indeed, we all thought we knew the meaning of the term "transmitter substance". Synaptic transmission was considered to be a straightforward process that involved (i) the evoked quantal release of one particular organic compound from one or more nerve terminals, and (ii) the reaction of these transmitter molecules with specific receptor molecules embedded in the subsynaptic membrane, a reaction which, in turn, causes (iii) the transient opening of certain "ionic channels", leading to (iv) "inhibitory" or "excitatory" postsynaptic potentials.

This happy state of innocence is definitely over. The number of potential transmitters has increased explosively since it was recognized that peptides can function not only as hormones (or neurohormones) but as transmitter substances. Indeed, the boundaries between "classical" transmitters and neurohormones, between "classical" synaptic transmission and neurosecretion become obsolete as the concept of "neuromodulation" assumes its current prominent role. We may as well employ the term "transmitter substance" (or simply "transmitter") to denote any compound released from a nerve cell which in one way or another affects the excitability of one or more neighbouring cells.

The recognition of "spontaneous" quantal and non-quantal transmitter release has added a new dimension to the role of nerve terminals. Even in the absence of nerve impulses, the liberation of transmitter can affect cellular excitability. This "spontaneous" transmitter release is subject to what we now refer to as "modulation"; that is, to chemical control. Not only the subsynaptic membrane responds to transmitters, but extrasynaptic membrane areas as well and to these belong those of the nerve terminals themselves from which transmitter is

being released. The classic concept of "chemical transmission" is now being supplemented with the new concept of non-synaptic transmission, and of neuromodulation.

This complex subject matter is in urgent need of systematization. A critical evaluation of the Symposium must take cognizance of the general situation of the field of neurobiology already alluded to, but must also refer to a wider range of specific points:

(i) It has become fashionable to refer to invertebrate species as "primitive" animals and to look at them as some sort of precursors of vertebrates. Indeed, it is not uncommon to find even insects considered to be ancestors of vertebrates! No doubt, the vertebrates evolved from invertebrates, but certainly they did not originate from any of the currently living animal groups. Insects as well as gastropods are at the end of evolutionary lines; there is no evolutionary link from insect or from gastropod to mammal - except by way of common ancestors that looked neither like insects nor like gastropods.

(ii) It is another fad to refer to one's experimental animal as a "model". Such terminology may be expedient within the framework of grantsmanship but it is unworthy of the ideals of comparative physiology in which each species is a worthy object by itself. For the comparative physiologist, the function of each organ and of each cell is interesting because of its adaptedness to the particular life style of that species. It is these special features of a general mechanism that makes research on a given species so especially exciting. The notion of a "model" implies that the animal is a model of something else. Are invertebrates models of vertebrates? Can the nervous system of Aplysia really be a model of a mammalian brain? Can a crayfish neuromuscular synapse be a model of a generalized synapse? Indeed, in the context of General Physiology, every real natural system is a model for an abstract, generalized system. Biology would be very poor if it would restrict its outlook to that of a general science, if physiology would be identified with general physiology. And neurobiology would lose much of its excitement and challenge if it restricted its activities to that of a general neurobiology. The true wealth of neurobiology

lies in its comparative aspect, in the recognition of the tremendous range of structural and functional adaptations and specializations.

(iii) The new research techniques often make us forget the biological or even the physiological context in which the results obtained ought to be placed. It is easy enough to state the results obtained with a particular technique but what is their physiological significance? How do the new findings help us to understand the life style of the particular animal we have studied in such detail? Can our knowledge of the life of the whole animal help to extract meaning from our new data? We must dare to supplement the question "how?" with the question "why?"!

(iv) Too often are we satisfied when we are able to fit our research results to a fashionable general term. "Modulation" is such a popular term, in spite of the fact that it is rarely given precise definition. We loosely employ such words as "neuromodulator" or "modulator substance" without bothering to ask what is the functional significance of the particular "modulation". We hardly ever ask what actually is being modulated.

If the action of a given organic compound does not exactly fit the classic concept of transmitter substance, it is deemed satisfactory to suggest that it might play the role of a modulator, leaving the task of recognizing the significance of such a statement to others who, perhaps, know what this term implies. Neurobiologists ought to go beyond such generalizing statements. We know enough of synaptic transmission to formulate a program of analysis of modulatory action. If indeed a modulator substance affects synaptic transmission, we ought to look for its effects on (a) transmitter synthesis, (b) mobilization, (c) release coupling (excitation-secretion coupling), (d) spontaneous transmitter release, (e) receptor affinity, (f) number and density of receptors, (g) channel activation and inactivation kinetics, (h) number and density of channels, (i) ionic selectivity of channels, (j) the coupling between receptor and channel, (k) stimulation or inhibition of "second messenger" systems, (l) the kinetics of transmitter inactivation by enzymes and uptake carriers, and (m) the kinetics of

transmitter (re-)uptake. In the context of recent findings, this list can be extended further to include research into (n) the modulation of facilitation (or antifacilitation) of transmitter release, and of (o) the relative proportion of the different transmitter substances released from the given terminal.

(v) We loosely employ the terms "inhibition" and "excitation", presuming that the state of inhibition is one of diminished probability of firing, while excitation is a condition of increased "excitability", meaning an increased probability that the cell in question generates an action potential. In the light of what is now known of neuronal function, such a motion is outmoded. Excitation might well represent a state of increased metabolic activity, and this state might be accompanied by the electrophysiological signs of increased membrane conductance for Cl^- and/or K^+, the "classical" symptoms of "inhibition".

(vi) Neurotransmitters and pharmacological agents acting as agonists and antagonists are nowadays assumed to interact with more or less specific membrane-bound receptor molecules, generally referred to simply as "receptors". This assumption is often based on the fact that the kinetics of the action of these substances obeys formal equations of enzyme kinetics. It must be recognized, however, that this formalism is satisfied also by other kinds of molecular events. It is curious that the action of anaesthetics on cells and cell membranes is rarely interpreted in terms of receptors for these anaesthetics. It is all too often forgotten that there have been very successful hypotheses which employ processes that differ from the receptor concept. Much of our limitation of the current conceptual framework comes from an oversimplified scheme of cell and membrane structure: most images of cell membrane structure ignore the otherwise obvious fact that these membranes do not exist in empty space but that they are bounded by an aqueous medium which imparts to both membrane surfaces an additional structural feature of adsorbed ions and water. Any substance that disturbs these superstructures will alter membrane behaviour. There is impressive experimental evidence that

many of the pharmacologically active substances disturb the structural organization of membrane-adsorbed water, alter the phase-boundary potential, the solubility coefficient or the partition coefficient of certain inorganic ions at lipid-water interphases. As the known number of "transmitters" that can act on a given limited membrane area increases, the notion that all of them interact with specific receptors becomes absurd. Nerve terminals of less than a micrometer diameter are supposed to possess, in addition to various carriers, adrenergic alpha and beta receptors, muscarinic and nicotinic cholinoreceptors, and various amino acid as well as peptide receptors. It is hard to believe that so restricted a membrane area can be occupied by so many large molecules.

(vii) It has become fashionable to discover the type of ionic channel that is being activated (or inactivated) by a given transmitter in a given membrane. This "channel game" reminds one of the "particle game" of atomic physicists. Every year new channels are being postulated, indeed one must wonder, how it was possible to obtain any clean results implying simple K^+, Na^+ or Cl^- channels, before all the currently hypothesized channels were found. Ionic specificities and gating mechanisms of such channels are usually explored with the technique of ion substitution. Rarely is attention being paid to the "side effects" of the alteration of the ionic environment of membranes and their channels. Removal of calcium from the external medium not only alters the calcium gradient across the membrane but also interferes seriously with normal membrane structure and stability. Indeed the change of activity of any ionic species can be expected to affect membrane behaviour, and with it the behaviour of ionic channels and, of course, that of membrane receptors. We simply cannot alter just one variable without affecting some or even all others. Substitution of inorganic by organic ions, of chloride, for instance, by propionate, not only alters chloride concentration but also affects intracellular pH and osmoticity. And, speaking of organic anions: pharmacological studies on neurons of certain invertebrates such as leeches rarely pay attention to the fact that in these animals a large percentage of extracellular anions is composed of or-

ganic anions and that an entirely inorganic saline medium grossly alters the natural conditions. Generalizations can be dangerous!

(viii) Immunocytochemistry can produce fantastically beautiful pictures. Selective staining or fluorescence of neurons can be very deceptive, however. Antibodies are not necessarily specific for a given presumed transmitter substance; in the case of neuropeptides, they may simply react with a certain amino acid sequence and this may be present in any number of peptides or proteins. Rarely do authors report on adequate controls of the specificity and selectivity of their antibodies. Indeed, conditions may be such that the absence of a reaction does not prove the absence of the substance one is seeking to detect! This is particularly true when antibodies against enzymes (like cholinesterase or dopa-decarboxylase) are used. Even if the prosthetic groups are alike, the complex tertiary and quaternary structure of the enzyme molecules may well differ in different species - even in different cell types.

Unless immunocytochemistry is coupled with bioassays and other more specific chemical, pharmacological, and physiological tests, the results are at best suggestive. Unless the functional role of immunoreactive cells has been established, immunocytochemical data alone have little more than aesthetic value.

(ix) It is generally assumed that transmitter substances are released only from nerve terminals. We ought to remember, however, that there was a time when neurosecretion was shown to occur from the soma of neurosecretory cells. Indeed, if we are to believe the immunocytochemical and fluorescence pictures, transmitters do indeed occur throughout the entire neuron in fairly similar concentration. Is the presence of transmitter an indication of its releasability from the site where it is found? If it is not released wherever it occurs, how do we know that the particular cell releases the detected compound at all?

(x) We have become accustomed to the notion that the nervous system is an assembly of neurons. So-called "nerve stains" yield deceptively beautiful pictures of neuronal networks, but they obscure the fact that nervous systems consist not only of

neurons. The glial elements, although much neglected, represent a major cellular compartment, and most nervous systems are richly vascularized. What do we know of glial-neural interactions, what do we know about neurovascular and glial-vascular interactions? Neurobiologists, by and large, design their experiments as if the neurons were suspended in empty space - or at least in a structureless aqueous medium. We have much to learn indeed! A look at any electron-micrograph can convince us that the space between neurons is filled with cellular entities. At least once in a while we ought to look at such pictures!

As this Symposium draws to a close, we may let the many reports and discussions pass review. Do we recognize signs of the ten problem areas I have just outlined? We will certainly take home many lessons, the most important one, however, will be that we have much to learn and that neurobiology is the most challenging field of biology. Let us not forget that whatever research method we are using, we are studying living creatures, and that our investigations must be designed to provide a deeper understanding of animal function and behaviour than we currently command. Transmitters, modulators and receptors will continue to haunt us for many years to come, and as time moves on we will certainly come closer to giving these concepts a more precise meaning. This wonderful world of invertebrate animals will continue to fascinate us and to challenge our curiosity. The results of our labours can only be an enrichment of our experience of this marvelous life on earth. We look forward to sharing our new discoveries in the not too distant future!

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